Adv Biochem Eng Biotechnol
DOI: 10.1007/10_2016_47

Reporter Gene Assays in Ecotoxicology

Tal Elad and Shimshon Belkin

Abstract The need for simple and rapid means for evaluating the potential toxic effects of environmental samples has prompted the development of reporter gene assays, based on tester cells (bioreporters) genetically engineered to report on sample toxicity by producing a readily quantifiable signal. Bacteria are especially suitable to serve as bioreporters owing to their fast responses, low cost, convenient preservation, ease of handling, and amenability to genetic manipulations. Various bacterial bioreporters have been introduced for general toxicity and genotoxicity assessment, and the monitoring of endocrine disrupting and dioxin-like compounds has been mostly covered by similarly engineered eukaryotic cells. Some reporter gene assays have been validated, standardized, and accredited, and many others are under constant development. Efforts are aimed at broadening detection spectra, lowering detection thresholds, and combining toxicity identification capabilities with characterization of the toxic effects. Taking advantage of bacterial robustness, attempts are also being made to incorporate bacterial bioreporters into field instrumentation for online continuous monitoring or on-site spot checks. However, key hurdles concerning test validation, cell preservation, and regulatory issues related to the use of genetically modified organisms still remain to be overcome.

Keywords Bacterial bioreporter, Biosensor, Dioxin, EDC, Environmental toxicology, Genetic engineering, Genotoxicity, Reporter gene, Stress response, Toxicity, Whole-cell bioassay

Contents

T. Elad and S. Belkin (✉)
Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel
e-mail: shimshon.belkin@mail.huji.ac.il

1 Introduction

When attempting to decipher the effect of a sample of interest on living organisms, an environmental toxicologist has at his disposal a tool kit with a choice of bioassays, which may be broadly categorized into two groups. The first is aimed at quantifying the general toxicity of the studied sample based on its effect on a test organism. Common general toxicity bioassays are based on higher organisms, mainly fish and crustaceans, including the standardized zebrafish [1, 2] and *Daphnia magna* acute toxicity tests [3]. The second group of bioassays allows for the detection of compounds with a defined effect at the molecular level, as pioneered by the *Salmonella* Mutagenicity Test [4]. Integral to effect testing are reporter gene toxicity bioassays, which utilize cells that respond to environmental stimuli through the synthesis of reporter proteins. In this chapter we explain the concept of reporter gene toxicity bioassays, review past and current advances, and discuss future prospects, mainly focusing on bacteria-based systems.

2 Microbes as Toxicity Test Organisms

An interesting characteristic of ecotoxicology is that whereas, on the one hand, there exists a set of long-established toxicity testing procedures, standardized, validated and in routine use, on the other hand, there is a continuous stream of proposed new bioassays. The latter are designed to address new classes of chemical challenges, employ different test organisms, or generally improve our ability to protect both the environment and human health from the presence of deleterious compounds. Although many of these assays remain as unrealized proposals in the scientific literature, others proceed all the way to full standardization, validation, and regulatory acceptance.

An important group of test organisms often found among such publications is bacteria. Although a direct relevance to human health does not always exist, bacteria

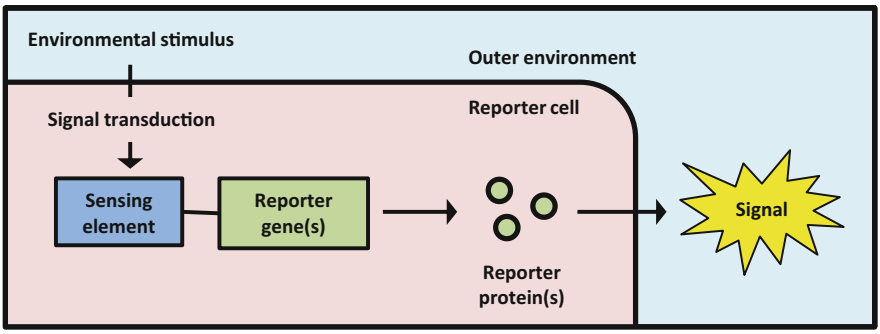

Fig. 1 Schematic depiction of a microbial bioreporter. A host cell is transformed with a fusion of a reporter gene(s) to a gene promoter (sensing element) known to be activated by a specific stress or chemical. Under the relevant environmental conditions, the resulting construct synthesizes the reporter protein(s), producing a readily quantifiable dose-dependent signal

are nevertheless endowed with several characteristics that render them highly attractive test organisms: they are fast to respond, they can easily and cost-effectively be obtained as large and homogeneous populations of live cells, and they can be preserved for prolonged periods of time and made available at short notice. These advantages, realized several decades ago, has prompted the development of the now widely accepted luminescent bacteria test, in which the short-term decrease in light emission by the naturally bioluminescent marine bacterium *Aliivibrio fischeri* (previously known as *Vibrio fischeri*) serves as an indication of sample toxicity [1, 2].

Another important advantage of bacteria as test organisms is their easy amenability to molecular engineering. The fusion of inducible gene promoters to diverse reporter genes, originally employed as molecular laboratory tools for studying gene regulation and expression, has found a different niche in the detection of biological effects exerted by the environment and sensed by the cells. The combination of a sensing element (in most cases a gene promoter), known to respond to a specific environmental condition, and a reporting element, the activity (or presence) of the product(s) of which can be easily monitored in real time, opens a broad spectrum of options for the design and construction of what are now often referred to as bacterial bioreporters. These can be tailored to recognize and detect specific chemicals, groups of chemicals, or deleterious global bio-effects such as toxicity or genotoxicity. The latter group is at the focus of the present review (Fig. 1).

3 Molecular Engineering of General Toxicity Bioreporters

As indicated above, the design of a toxicity bioreporter calls for an a priori selection of two major building blocks: a sensing and a reporting element. The reporting elements available comprise a relatively short list of options; most of these genes encode enzymes *the activity* of which produces a physical signal, whereas a few

code for proteins, *the amount* of which can be assayed in real time. Fluorescent proteins, most notably green fluorescence protein (GFP) and its various derivatives, are the most prevalent representatives of the second group. Among the enzyme-encoding reporter genes, the most commonly used are *lacZ* (β-galactosidase) and *luxAB* (bacterial luciferase). The nature of the physical signal emitted depends both upon the reporter enzyme and, in some cases, its substrate. LacZ, for example, can be made to generate a substrate-dependent fluorescent, luminescent, or chromogenic signal, according to the designer's wishes. The same enzyme, if provided with a substrate the product of which is electrochemically active, can also generate an electrochemical signal, thus alleviating the need for optical detection equipment. Reporter genes commonly employed in bioreporter design have been listed in several comprehensive reviews [5–7].

Whereas, as noted above, the available selection of reporter genes is relatively limited, the options for gene promoters acting as the sensing element of the bioreporter are practically unlimited, and it may be stated with confidence that a sensing element can be found for any chemical or group of chemicals exerting a biological effect on live bacterial cells. Bacterial bioreporters that can sensitively and specifically detect polycyclic aromatic hydrocarbons such as naphthalene [8] or heavy metals such as mercury [9] were first described over 20 years ago. Since then many reports have described the construction and characterization of bacterial bioreporters for the detection of diverse targets including additional heavy metals [10, 11] and aromatic compounds [12, 13], nutrients [14, 15], and traces of explosives [16, 17].

For the detection of general toxicity, however, the issue becomes more challenging as gene promoters need to be identified that will respond not to specific chemicals but rather to general toxicity. Such genes should be induced in response to an imbalance in cellular homeostasis, and their degree of activation should represent the overall toxicity level. Over the years, several bacterial constructs have been reported to be characterized by relatively broad response spectra, among them those based on the promoters of the *Escherichia coli* genes *grpE* [18] and *fabA* [19] as sensing elements. These genes, involved in the regulation of the bacterial heat shock response and fatty acid synthesis, respectively, were shown to be induced by diverse toxic compounds belonging to different chemical groups. Other gene promoters reported to be of a relatively broad response spectrum are *uspA* [20] and *micF*. The latter, demonstrated to be induced by superoxide radicals [21], serves as an excellent indication of the involvement of oxidative stress in the toxic effects of numerous chemicals.

Regardless of how sophisticated is the molecular engineering, it is unreasonable to expect that any single bioreporter will be able to detect the deleterious effects of the entire spectrum of toxic compounds of interest, which may be of highly varied chemical natures and toxicological characteristics. The obvious solution to this limitation is the use of a panel of reporter strains, each responsive to a different class of compounds, which together provide a much better coverage of the relevant threat lists. Several such panels have been reported, varying in the number of their members from 5–14 [21–28] to several dozen [29] to genome-wide collections covering the entire *E. coli* promoter set [30]. Importantly, in these cases, advantage

is taken of the fact that a panel's response not only indicates the existence of a toxic threat but also provides information concerning the toxic and chemical nature of the sample contents, as may be derived from its response pattern.

Moreover, comprehensive libraries of reporter gene fusions can offer an alternative to the more laborious and more expensive DNA microarray and RNA sequencing (RNA-seq) techniques for genome-wide transcriptional analysis [31, 32]. In particular, they can be used for assessing the toxicity and for determining the mode of action of known and potential environmental pollutants through gene expression, as proposed in a series of publications, in which mercury [33], nanomaterials [34], naphthenic acids [35], triphenyltin chloride [36], hydroxylated polybrominated diphenyl ethers (OH-PBDEs; [37]), bisphenol A [38], and flame retardants [39, 40] were tested against arrays of dozens to thousands of different bacterial bioreporters. Also explored in this context was the opportunity to account for the cells' dynamic response and to look beyond predefined endpoints [41].

4 Genotoxicity Bacterial Bioreporters

A special position among toxicity testing constructs is held by those tailored to detect the potential DNA damage hazards inherent in the sample, and thus assess its mutagenic and possibly carcinogenic risks. In the selection of sensing elements to be used for the construction of genotoxicity reporter systems, the most promising candidates are promoters of genes involved in DNA repair. Such genes, induced in response to either actual DNA damage or to the presence of DNA damaging agents, mostly belong to one of two inducible bacterial systems: the *recA*-dependent, *lexA*-controlled SOS response and the *recA*-independent, *ada*-controlled adaptive system induced in response to alkylation damage of DNA.

4.1 SOS-Based Assays

The bacterial SOS response is negatively controlled by the LexA protein that binds to the SOS box in the promoter region of the regulon genes, repressing their expression. De-repression occurs when the RecA protein binds to single-stranded DNA at replication forks blocked by DNA damage. Once bound to DNA, the RecA protein changes conformation and acts as a co-protease in the cleavage of LexA, thus allowing transcription of the SOS genes [42, 43].

The first commercial bacteria-based genotoxicity test kit, the SOS chromotest, was proposed in 1982 [44]. This test employs an *E. coli* strain with a transcriptional fusion between the SOS cell division inhibitor gene *sfiA* (also known as *sulA*) and the reporter enzyme β-galactosidase (LacZ). Oda and co-workers introduced the *umu* test, based on a *Salmonella typhimurium* strain harboring a *lacZ* fusion to the promoter of another SOS response gene, *umuC* [45]. Results from the *umu* test

correlated well with those from both the Ames test and the SOS chromotest, and were highly representative of rodent carcinogenicity [46, 47]. Following several modifications, the *umu* test achieved ISO standardization [48]. Another SOS gene promoter that has been integrated into a commercial product is that of *recN*, which is involved in double stranded DNA break repair. Known as the VITOTOX® test, this assay comprises *E. coli* and *S. typhimurium* strains, which harbor a fusion of the *recN* promoter to the *A. fischeri luxCDABE* gene cassette [49]. The VITOTOX® test strains were evaluated with a variety of chemicals and environmental samples [49–52].

Additional notable SOS genes, on the basis of which genotoxicity bacterial bioreporters were constructed, are *recA* and *cda*. As described, the RecA recombinase gene plays a key role in the SOS response by its co-protease activity on the LexA repressor. Nunoshiba and Nishioka [53] described, and tested against an array of compounds, the colorimetric Rec-lac test, based on *E. coli* strain GE94 that carries a *recA::lacZ* fusion and its DNA repair-deficient derivative strains such as KY946 (*uvrA*), KY945 (*recA*), and KY943 (*lexA*). A different reporter system, the *A. fischeri luxCDABE* operon, was used by Vollmer et al. [54] to generate several *E. coli* reporter strains, one of them (DPD2794) carrying a *recA::luxCDABE* fusion. Bacterial bioreporter constructs of *recA* have since been widely used alone or with other reporter strains in diverse studies for toxicity assessment (e.g., [24, 27, 55, 56]). The colicin D gene *cda*, a constituent of the ColD plasmid [57], served as a basis for a bioluminescent genotoxicity sensor using *Photobacterium leiognathi luxCDABE* as a reporter. This SOS *lux* test responded to diverse genotoxic agents and was comparable in sensitivity to the SOS chromotest, the *umu* test, and the Ames test [58]. In their GenoTox version, *cda*-based genotoxicity bacterial bioreporters use *gfp* as a reporter gene [59]. The signal is measured by means of flow cytometry, which was reported to enhance the sensitivity of fluorescent bioreporters [60, 61]. Furthermore, the *cda-gfp* GenoTox assay exhibited lower detection thresholds than other genotoxicity reporter gene assays that use different promoter-reporter combinations [62].

4.2 Non-SOS-Based Assays

Several bacterial DNA protection and repair systems that are independent of RecA-LexA control have been described; one of these, most efficiently induced by alkylating agents, has been generally termed the adaptive response. The adaptive system responds to the presence of methylated phosphotriesters generated by DNA alkylation that activate the *ada* gene product which, in turn, triggers the transcription of genes such as *ada*, *alkA*, *alkB*, and *aidB* [63]. Vollmer et al. [54] used *alkA*, which encodes a repair glycosylase (*N*3-methyladenine DNA glycosylase II; [63]) for the construction of a bioluminescent reporter strain. The construct displayed a very strong response to the alkylating agent methylnitronitrosoguanidine (MNNG), the magnitude of which was enhanced by very low background bioluminescence.

Another non-SOS gene employed was *nrdA*, which encodes for a ribonucleoside diphosphate reductase and is strongly affected by DNA damage. An *E. coli* strain carrying a fusion between the *nrdA* promoter and the *Photorhabdus luminescens luxCDABE* operon responded to the DNA damaging agents nalidixic acid, mitomycin C, MNNG, 4-nitroquinoline 1-oxide (4-NQO), and hydrogen peroxide, but not to other oxidants or phenolic compounds [64]. Combined together, *sulA*-, *recN*-, *recA*-, *alkA*-, and *nrdA*-based luminescent bacterial bioreporters were shown to be able not only to detect genotoxic agents but also to classify their potential mode of action [65, 66].

5 EDCs and Dioxins

Endocrine disrupting compounds (EDCs), as well as dioxin and dioxin-like compounds (hereafter dioxins), are given special attention by monitoring agencies because of their potent negative impact on both human and environmental health. The mode of action of EDCs and dioxins is based on the translocation of an activated receptor to the nucleus. By definition, such a sequence of events can be directly detected only by use of eukaryotic bioreporters. Indeed, yeast and mammalian cells are central in the design of either EDCs or dioxins reporter gene assays.

The yeast estrogen and androgen screens (also known as YES and YAS, respectively) are prominent among yeast-based bioreporter assays for EDC detection [67, 68]. The *Saccharomyces cerevisiae* YES and YAS bioreporter strains are genetically modified to express the human estrogen receptor (hER-α) and the human androgen receptor, respectively. They are also inserted with a fusion of the *lacZ* reporter gene and human estrogen/androgen response elements, and, supplemented with a chromogenic substrate, yield a color product once a recognition event between receptor and compound occurs. The YES and YAS were tested with, and adapted to, various matrices, including dairy wastes [69] and aquifer samples [70], and are commercially available. The coupling of high-performance thin-layer chromatography with the yeast estrogen screen (planar-YES, p-YES) can serve as a fast and robust screening tool in effect-directed analysis, which links chemical analysis with effect testing [71].

Following the same guiding principle as the YES and YAS, similar yeast-based bioreporter assays have been developed by employing *gfp* [72, 73] and firefly luciferase [74] reporter genes. A *luxAB* bioluminescent modification was shown to be applicable for the United States Environmental Protection Agency's Endocrine Disruptor Screening and Testing Program [75], and most recently facilitated the comparison of treated wastewater from a traditional activated sludge facility and a membrane bioreactor [76]. Human cell line-based reporter gene assays are also at the forefront of EDC detection. Such are the transactivation in vitro assays using the hERα-HeLa-9903 human cervical tumor and the BG1Luc-4E2 human ovarian adenocarcinoma cell lines. These two cell lines express ER and have been stably transfected with an ER-responsive luciferase reporter gene, and the two

assays are accredited in the OECD Guidelines for the Testing of Chemicals [77]. Widely used are the ER CALUX® strains, which include human bone marrow and breast cancer cell lines incorporating the firefly luciferase gene coupled to estrogen responsive elements [78].

Dioxins exert their adverse effect by activating the intracellular aryl hydrocarbon receptor and transcription factor (AhR) of all vertebrates, including humans [79]. The major reporter gene assays for dioxin detection accordingly utilize mammalian cells and measure AhR-dependent gene expression. The DR CALUX® bioassay, for example, consists of a genetically modified H4IIe rat hepatoma cell line that contains the firefly luciferase gene fused downstream to AhR response elements [80, 81]. The DR CALUX® was used for screening of feed and food samples in official control programs [82], and was also validated against chromatographic analytical methods [83] and calibrated in intra- and inter-laboratory studies [84]. A somewhat different variation of the reporter gene assay concept is manifested in the H4IIe rat hepatoma ethoxyresorufin-*O*-deethylase (EROD) bioassay, in which the cytochrome P450 enzyme CYP1A1, the expression of which is AhR-dependent, serves as an endogenous reporter gene, de-ethylating its substrate 7-ethoxyresorufin to an easily measured fluorescent product, resorufin [85]. Although the EROD bioassay is considered rapid and simple, its sensitivity might be hampered by the inhibiting effect of AhR ligands on CYP1A1 [86]. In addition to H4IIe, a diverse range of cell lines has been employed for the development of AhR-based bioassays by use of either transfected reporter genes (e.g., luciferase, *gfp*, *lacZ*) or CYP1A1 as an endogenous reporter (see [87] for a review). To circumvent the difficulty inherent in harnessing prokaryotes for the detection of dioxin-like compounds, Turner et al. [88] have introduced an *E. coli* luminescent recombinant strain for the detection of hydroxylated polychlorinated biphenyls (OH-PCBs) by taking advantage of the recognition of a group of related compounds, namely hydroxylated biphenyls, by the product of the *hbpR* gene in the *hbp* operon from *Pseudomonas azelaica* HBP1.

6 From Bioassays to Biosensors

A biosensor is commonly defined as a device that combines a biological entity and an electronic system for the detection of various substances in water, air, or body fluids [6]. A capacity to withstand environmental conditions, easy handling, rapid responses, and readily measurable signals make bacterial bioreporters highly suitable for the development of biosensors for field applications. A bacterial bioreporter field biosensor should provide the conditions for reporter cell long term preservation, and be comprised of a signal transducer, signal processing and analysis algorithms, and, if required, a communication unit. Although a fully operational bacterial bioreporter biosensor embodying this entire wish list has not yet been introduced commercially, substantial steps towards its introduction have been

taken. Some examples are described below, organized into two categories: online continuous monitoring and portable field kits.

6.1 Online Continuous Monitoring

Online continuous water quality monitoring is normally based on the continuous measurement of physicochemical parameters such as temperature, pH, conductivity, and oxygen, and is often supplemented by automated sampling for specific analysis of organic and inorganic pollutants, performed using laboratory-based chromatographic techniques. Because of the elaborate sample preparation procedures and high costs, the time resolution of the data provided by chromatography-based continuous monitoring is low; in most monitoring programs a daily sampling frequency is considered high [89]. Furthermore, it is difficult for this approach, geared towards the analysis of specific chemicals, to detect unknown compounds. As a result, there is a risk that sporadic contamination is not identified on time. As a partial solution to this need, online continuous biomonitoring systems have been developed to provide a rapid alarm against the presence of contaminants. These biological early warning systems are based on the monitoring of behavioral or physiological stress responses of test organisms that are continuously exposed to the tested water either in bypass systems or directly in-stream. The test organisms range from fish [90], daphnia, and mussels [89, 91, 92] to algae and bacteria [93].

Bacterial bioreporters offer simple and rapid online continuous biomonitoring solutions that can indicate not only the occurrence of toxic events but also the specific effect of the chemicals involved. Such designs mostly include luminescent reporting elements, which require neither the addition of exogenous reagents nor the hardware for optical excitation. Gu and co-workers [94, 95] have developed a multichannel system featuring stress-responsive reporter strains kept in suspension in a steady physiological state. Each channel was assigned with a reporter strain responsive to a specific kind of stress, and the response profile was used for pollution classification. To avoid the complexity inherent in maintaining a continuous culture [96], cells have been immobilized on the tips of a liquid light guide [97] or an optical fiber [98, 99]. Immobilized reporters have also been loaded onto a disposable card, which was employed in the assembly of a multi-strain biosensor for online detection of metal pollution and its classification [100]. Another report described the construction of a biosensor for online continuous water monitoring, at the heart of which was a panel of luminescent bacterial reporter strains immobilized in specialized flow-through chambers and probed by sensitive light detectors [101]. The living cell panel consisted of a general toxicity reporter (*micF-lux*), a genotoxicity reporter (*recA-lux*), and a heavy metal reporter (*arsR-lux*). The biosensor was shown to operate for at least 10 consecutive days under continuous tap water flow, detecting and classifying spikes of both model chemicals and industrial wastewater within 0.5–2.5 h, and requiring minimal maintenance. The detection

was facilitated by a signal processing procedure laid out to determine whether an observed increase in the light intensity is significant enough for raising the alarm on a possible pollution event. The procedure was centered on the rate of change of the light signal, which was found to be a better indicator of reporter induction than the signal's absolute value; basal light signal fluctuations were also considered to eliminate false positives (Fig. 2).

6.2 Portable Field Kits

Easy-to-operate field kits for on-site monitoring allowing real-time analysis is beneficial when a rapid response is required or in remote areas where equipped laboratories or skilled personal are scarce. In an attempt to realize the substantial potential of microbial bioreporters to be incorporated in to such instrumentation, researchers have proposed and demonstrated the feasibility of various portable biosensor configurations, adopting diverse reporting systems and preservation techniques. Representative examples follow.

Nivens et al. [102] pioneered the field by describing a Bioluminescent Bioreporter Integrated Circuit (BBIC), a setup designated for field deployment consisting of bioluminescent bacterial bioreporters sustained within a micro-environment, a complementary metal oxide semiconductor (CMOS) integrated circuit microluminometer, and a light-tight enclosure. Equipped with the bacterial bioreporter strain *Pseudomonas fluorescens* 5RL, the BBIC setup was reported to detect low concentrations of model compounds salicylate and naphthalene in liquid and gas environments, respectively [102, 103]. Eltzov et al. [104] assembled a portable biosensor integrating a PMT detector and a liquid light guide. For testing, the biosensor was equipped with disposable calcium alginate pads containing three stress-specific recombinant reporter strains, harboring a plasmid-borne fusion of *recA*, *grpE*, or *fabA* to the *luxCDABE* gene cassette. The biosensor was then exposed to organic solvents, heavy metals, and endocrine disrupting compounds, and to environmental water samples, and its capability to detect the presence of toxicants was confirmed. Notably, the group demonstrated that the same biosensor can also be used for indoor air toxicity monitoring [105].

Using β-galactosidase reporter protein and *E. coli* hosts, Stocker et al. [106] have developed a paper strip test. According to the described protocol, reporter cells are suspended in drying protectant solution and spotted on paper strips, which are in turn vacuum-dried and stored. Upon use, the strips are placed inside the sample for 30 min. A substrate (X-gal) solution is then spotted on the bacterial spot and the color that develops is compared to that of a reference test. Originally demonstrated for rapid and quantitative measurements of arsenic in potable water, this paper strip whole-cell biosensor was later also successfully tested with quorum signaling molecules [107].

Daunert and co-workers have incorporated spore-forming bioreporters into a centrifugal compact disk (CD) microfluidic platform towards the development of a

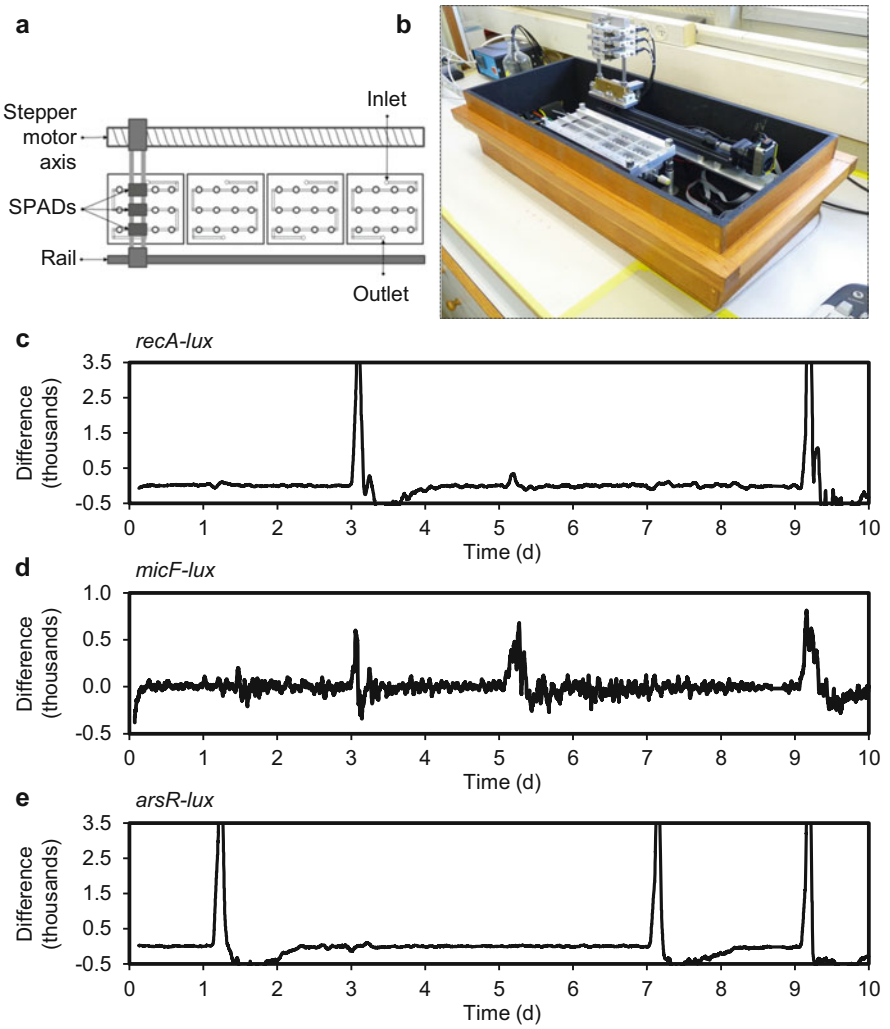

Fig. 2 A bacterial bioreporter-based biosensor for online continuous monitoring of water toxicity. The biosensor consists of four separate flow-through chambers. Each chamber comprises 12 wells, in which reporter cells are immobilized in agar. Three aligned single-photon avalanche diodes (SPADs), connected to a single-axis linear stepper motor, measure the light signal at 1-min intervals. Each of *recA*-, *micF*-, and *arsR-lux E. coli* reporter strains was immobilized in an individual flow-through chamber in 12 replicates. Tap water was pumped continuously through the system for 10 days, in the course of which five simulations of pollution events were carried out by 2-h pulses of tap water spiked with arsenite (day 1 and day 7), nalidixic acid (a genotoxic agent; day 3), paraquat (an oxidative herbicide; day 5), and a mix of all three (day 9). (**a**) A partial scheme of the four biochips and the photodetectors. (**b**) A photograph of the system. (**c**–**e**) The recorded signals of *recA-lux*, *micF-lux*, and *arsR-lux* reporters, respectively; the y-axis represents the difference between two consecutive photon counts [101]

portable sensing system for fast on-field analysis [108]. The platform consists of reagent reservoirs controlled by burst valves, a mixing channel, and a detection chamber, which are microfabricated on a circular disk. Bacterial bioreporter spores, samples, and, if needed, a substrate solution, are placed in the reagent reservoirs. At a specific rotation frequency, the valves controlling the reagent reservoirs burst open and the fluids from the reagent reservoirs flow to the mixing channel and then to the detection chamber. The action of the platform was demonstrated by use of *lacZ* and *gfp* spore-forming *Bacillus spp.* reporter strains, which germinated and responded to their target analytes within 2.5–3 h. The advantages inherent in the ruggedness of bacterial spores hold promise in providing a biosensor with a long shelf life. Indeed, the same group reported that the sensing spores could be effectively stored for over 2 years at room temperature and for at least 1 year under extreme conditions [108–110]. Centrifugal CD microfluidics is an active area of research and development; for more information on the state-of-the-art thereof the reader is directed to a recent critical review [111].

Roda et al. [112] have developed a portable device comprising a disposable microwell cartridge with immobilized microbial bioreporters placed in contact with a compact CCD sensor through a fiber optic taper. Cells were immobilized in an aqueous mixture of agarose, PVP, and collagen and kept their vitality for at least 1 month at 4 °C. To demonstrate the biosensor's capability, the authors used, among others, a new yeast bioreporter, which emits both green light in response to androgens and red light as vitality control. Light signal separation was carried out by using printed filters, and testosterone was quantified after correction for cell vitality. The same group has also developed a cell phone-based biosensor by transforming a smart-phone, low-cost 3D-printed adapter, cell cartridges, and a specially-programmed application for bioluminescence detection into a standalone user-friendly platform for quantitative toxicity assessment [113]. A proof-of-concept was provided by the use of genetically engineered human cells that constitutively express a powerful green-light-emitting luciferase mutant.

Biran et al. [114] have constructed a bacterial bioreporter in which the tightly controlled strong promoter of the *E. coli* SOS response gene *sulA* was fused to the alkaline phosphatase-coding *phoA* reporter gene. Together with the design of a portable electrochemical detection platform [115], a chip able to detect DNA-damaging agents when dipped in water is envisaged. Tsai et al. [116] have introduced LumiSense, a portable system combining luminescent bacterial bioreporters loaded on a biochip and a linear charge-coupled device (CCD), the protective window of which was removed for high light collection efficiency. The system picked up a dose-dependent signal from a reporter strain harboring a fusion of the *recA* promoter and the *luxCDABE* gene cassette as the latter was exposed to various concentrations of nalidixic acid.

Choi and Gu [24] combined into a portable field kit a small light-proof test chamber, an optic-fiber, a luminometer, and stress-responsive bacterial bioreporters, harboring *fabA*::, *grpE*::, *recA*::, and *katG::luxCDABE* fusions, freeze-dried within vials. When the kit was challenged with phenolic compounds, general toxicity was reported by the *fabA* and *grpE* bioreporters. The ARSOlux

biosensor test kit, developed by scientists at the Helmholtz Centre for Environmental Research—UFZ (Germany) and the Université de Lausanne (Switzerland), is based on lyophilized arsenic-specific luminescent reporter cells. Bioreporter vials are injected on site with the sampled water, incubated for 2 h, and arsenic concentrations are inferred by measuring the bacterial responses using a portable luminometer. The system, successfully tested in Bangladesh and Argentina, requires no preparatory steps except for filtration of turbid samples [117, 118].

7 Performance Enhancement

Bacterial bioreporters are not only carriers of a simple promoter-reporter fusion but can also be subjects of additional genetic manipulations aimed at enhancing their performance. Such manipulations have taken several forms, including the introduction of mutations into the tester strains, the modification of the sensing or reporting elements, and, for genotoxicity detection, the incorporation of metabolic activation capabilities.

7.1 Introduction of Mutations into Host Tester Strains

Several mutations introduced into bacterial bioreporters have been reported to lower their detection thresholds. Mutations of *uvrAB* and *rfa*, which hinder DNA repair and increase membrane permeability, respectively, render genotoxicity bacterial bioreporters responsive to lower concentrations of certain genotoxic agents [4, 44, 45, 51, 53, 59, 114, 119, 120]. A similar effect on reporter gene assays for genotoxicity detection is achieved by *tag* and *oxyR* mutations; the former inactivates 3-methyl-adenine DNA glycosylase I, an enzyme involved in the removal of alkylated bases from DNA, and thereby lowers the tester strain's detection thresholds towards alkylating agents, and the latter suppresses the oxidative stress response under the control of OxyR transcription regulator, increasing sensitivity to peroxide-generating compounds [46]. Last in this set of examples, limiting the host cell's efflux capacity by a *tolC* mutation lowered the detection thresholds of *cda*-, *recA*-, and *umuD*-based genotoxicity bioreporters [120–123] and those of *grpE*- and *fabA*-based general toxicity sensors [18, 19].

7.2 Modifying the Sensing or Reporting Elements

Molecular manipulations of the DNA fragment harboring the sensing promoter element to improve the bacterial response to target chemicals have been described. The influence of addition/subtraction of *lexA* binding sites on reporter gene

expression in the SOS promoter::*uidA* fusion under genotoxic stress was examined with several SOS promoters (*umuD*, *sulA*, *recA*, and *recN*; [124]). The highest signals were produced by constructs that either harbored an additional *lexA* binding site in the *sulA* promoter that overlapped the −35 promoter region or lacked one of the two *recN* LexA binding sites. Replacing the wild-type −35 promoter sequence with the −35 sequence of the highly active *trp* gene promoter improved the performance of a *umuDC*::*gfp* construct [125].

Changing specific nucleotides in specific positions using site-directed techniques is also efficient in improving bioreporter abilities. The VITOTOX® *S. typhimurium* bioluminescent genotoxicity tester strain was enhanced by an up promoter mutation where a consensus nucleotide was introduced in the *recN* promoter −35 region [49]. Similarly, the −35 element of the *sulA* promoter in a *sulA::luxCDABE* fusion was changed to a consensus sequence, improving light signal intensity, response time, and detection threshold [126].

An opposite approach to the precisely planned point mutations targeting nucleotides at specific positions is random mutagenesis in a directed evolution process. This approach is generally based on error-prone polymerase chain reaction (PCR), with or without the combination of a DNA shuffling procedure, which can be performed on the target DNA sequence; the resulting library of variants is then screened for the desired feature and selected isolates are reprocessed in the same manner. This procedure is often applied for modifying protein sequence and performance, but it may just as well be applied to regulatory areas of the bacterial genome. In one cycle of an error-prone PCR performed on the *sulA* promoter in a *sulA::luxCDABE* reporter, an improved mutant was isolated, harboring three point mutations relative to the wild-type sequence [126]. Interestingly, these mutations were not located within well-defined regulatory sequences. This finding demonstrates the potential power of the directed evolution process, which introduces mutations into random locations that would not necessarily be identified as obvious targets by bioinformatic tools.

Reporting elements have also been modified with the aim of enhancing bacterial bioreporter performance. The *Photorhabdus luminescens luxCDABE* cassette was rearranged and split into two independent functional units: *luxAB*, coding for the luciferase enzyme, and *luxCDE*, coding for the reductase complex, which catalyzes the synthesis of the aldehyde necessary for the reaction [127]. The expression of each subunit was put under the control of either an inducible stress-responsive promoter or a synthetic constitutive promoter in both general toxicity and genotoxicity *E. coli* reporter strains. In all cases, the split combinations proved to be superior to the native *luxCDABE* configuration. The best combination was that of an inducible *luxAB* and a constitutive *luxCDE*, resulting in dramatically improved assay parameters and, at the same time, indicating that aldehyde availability may be limited when the five genes are expressed together. Another modification of the *P. luminescens lux* system reduced the half-life of these proteins, allowing for the monitoring of both the initiation and the termination of transcription in real time, thus upgrading circuit performance [128].

7.3 Incorporating Metabolic Activation Capabilities

Bacterial-based assays cannot carry out the complex biochemical reactions collectively known as metabolic activation, which take place mainly in mammalian liver cells, and in which some xenobiotics are transformed into genotoxic forms [129]. To overcome this limitation, rodent-derived cytochrome P450 (S9) preparations, which can convert susceptible compounds into active genotoxic entities, are included in the procedures of, for example, the Ames test and the *umu* test [4, 48]. To reduce at least partially the dependency of reporter gene assays on S9 extracts, several attempts have been made to genetically engineer bacterial cells to incorporate some of the enzymatic activities involved in the activation process of xenobiotics. These include the development of a series of *S. typhimurium* tester strains with high *O*-acetyltransferase (O-AT) and nitroreductase (NR) activities, which displayed increased sensitivity towards nitroarenes and aromatic amines [59, 130–133]. Such successful efforts clearly demonstrate the viability and potential of this approach; nevertheless, it should be remembered that the cyt P-450 complex is composed of a plurality of activities, the integration of which into a single reporter strain is highly challenging.

8 Outlook

The development of reporter gene assays and their application as ecotoxicological assessment tools have been prompted over the last decades by the need for simple and rapid methods for toxicity assessment and pollutant detection. New and innovative assays are constantly being proposed and tested in many institutions worldwide. The new reporter gene assays show great promise in broadening the detection spectrum and increasing the sensitivity offered by presently available techniques. Moreover, microbial bioreporters have been successfully integrated into biosensor hardware for both laboratory and field use. Based on this progress, it is tempting to envision a validated set of bioreporters, covering a range of biological effects and indicative as to the nature of toxic samples, routinely employed either as a part of an online early warning system or for spot checks, and serving as first-line safeguards of human and ecosystem health. However, for such assays and biosensor devices to be implementable in real-world applications, several key hurdles still need to be overcome.

First, most proposed reporter gene assays have not yet been validated in large-scale comparative studies against standard methods, nor have they been the subjects of inter-laboratory round-robin tests to determine their reproducibility. Second, additional progress needs to be made in keeping microbial bioreporters alive and active for prolonged periods of time; lack of long term preservation is often a major factor limiting the application of whole-cell biosensors. Third, strict regulations prohibit the use of microbial bioreporters outside laboratories because of concerns

regarding the risks involved in the use of genetically modified organisms (GMOs). Although highly efficient measures to prevent the escape of bioreporters from portable field devices or continuous monitoring units can be taken, they might not suffice under current legislation.

Acknowledgements T.E. acknowledges with gratitude the Eshkol fellowship from the Israeli Ministry of Science and Technology. Research was supported in part by the Minerva Center for Bio-Hybrid Complex Systems and by the EU FP7 project BRAAVOO.

References

1. ISO (2007) ISO 11348:2007 Water quality – determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (luminescent bacteria test). ISO, Geneva
2. ISO (2007) ISO 15088:2007 Water quality – determination of the acute toxicity of waste water to zebrafish eggs (*Danio rerio*). ISO, Geneva
3. ISO (2012) ISO 6341:2012 Water quality – determination of the inhibition of the mobility of *Daphnia magna* Straus (*Cladocera*, *Crustacea*) – acute toxicity test. ISO, Geneva
4. Ames BN, McCann J, Yamasaki E (1975) Methods for detecting carcinogens and mutagens with the salmonella/mammalian-microsome mutagenicity test. Mutat Res 31:347–364
5. Daunert S, Barrett G, Feliciano JS, Shetty RS, Shrestha S, Smith-Spencer W (2000) Genetically engineered whole-cell sensing systems: coupling biological recognition with reporter genes. Chem Rev 100(7):2705–2738
6. van der Meer JR, Belkin S (2010) Where microbiology meets microengineering: design and applications of reporter bacteria. Nat Rev Microbiol 8(7):511–522
7. Yagi K (2007) Applications of whole-cell bacterial sensors in biotechnology and environmental science. Appl Microbiol Biotechnol 73(6):1251–1258
8. King JM, Digrazia PM, Applegate B, Burlage R, Sanseverino J, Dunbar P, Larimer F, Sayler GS (1990) Rapid, sensitive bioluminescent reporter technology for naphthalene exposure and biodegradation. Science 249(4970):778–781
9. Selifonova O, Burlage R, Barkay T (1993) Bioluminescent sensors for detection of bioavailable Hg(II) in the environment. Appl Environ Microbiol 59(9):3083–3090
10. Corbisier P, van der Lelie D, Borremans B, Provoost A, Lorenzo V, Brown NL, Lloyd JR, Hobman JL, Csöregi E, Johansson G, Mattiasson B (1999) Whole cell- and protein-based biosensors for the detection of bioavailable heavy metals in environmental samples. Anal Chim Acta 387(3):235–244
11. Magrisso S, Erel Y, Belkin S (2008) Microbial reporters of metal bioavailability. Microb Biotechnol 1:320–330
12. Applegate BM, Kehrmeyer SR, Sayler GS (1998) A chromosomally based *tod-luxCDABE* whole-cell reporter for benzene, toluene, ethybenzene, and xylene (BTEX) sensing. Appl Environ Microbiol 64(7):2730–2735
13. Tecon R, Beggah S, Czechowska K, Sentchilo V, Chronopoulou PM, McGenity TJ, van der Meer JR (2010) Development of a multistrain bacterial bioreporter platform for the monitoring of hydrocarbon contaminants in marine environments. Environ Sci Technol 44 (3):1049–1055
14. Gillor O, Hadas O, Post FA, Belkin S (2002) Phosphorus bioavailability monitoring by a luminescent cyanobacterial sensor strain. J Phycol 38:107–115
15. Mbeunkui F, Richaud C, Etienne AL, Schmid RD, Bachmann TT (2002) Bioavailable nitrate detection in water by an immobilized luminescent cyanobacterial reporter strain. Appl Microbiol Biotechnol 60:306–312

16. Garmendia J, De Las Heras A, Galvão TC, De Lorenzo V (2008) Tracing explosives in soil with transcriptional regulators of *Pseudomonas putida* evolved for responding to nitrotoluenes. Microb Biotechnol 1:236–246
17. Yagur-Kroll S, Lalush C, Rosen R, Bachar N, Moskovitz Y, Belkin S (2014) *Escherichia coli* bioreporters for the detection of 2,4-dinitrotoluene and 2,4,6-trinitrotoluene. Appl Microbiol Biotechnol 98(2):885–895
18. Van Dyk TK, Majarian WR, Konstantinov KB, Young RM, Dhurjati PS, LaRossa RA (1994) Rapid and sensitive pollutant detection by induction of heat shock gene-bioluminescence gene fusions. Appl Environ Microbiol 60(5):1414–1420
19. Bechor O, Smulski DR, Van Dyk TK, LaRossa RA, Belkin S (2002) Recombinant microorganisms as environmental biosensors: pollutants detection by Escherichia coli bearing *fabA′::lux* fusions. J Biotechnol 94(1):125–132
20. Van Dyk TK, Smulski DR, Reed TR, Belkin S, Vollmer AC, LaRossa RA (1995) Responses to toxicants of an *Escherichia coli* strain carrying a *uspA′::lux* genetic fusion and an *E. coli* strain carrying a *grpE′::lux* fusion are similar. Appl Environ Microbiol 61(11):4124–4127
21. Belkin S, Smulski DR, Dadon S, Vollmer AC, Van Dyk TK, LaRossa RA (1997) A panel of stress-responsive luminous bacteria for the detection of selected classes of toxicants. Water Res 31(12):3009–3016
22. Ahn J-M, Kim JH, Kim JH, Gu MB (2010) Randomly distributed arrays of optically coded functional microbeads for toxicity screening and monitoring. Lab Chip 10(20):2695–2701
23. Ben-Israel O, Ben-Israel H, Ulitzur S (1998) Identification and quantification of toxic chemicals by use of *Escherichia coli* carrying *lux* genes fused to stress promoters. Appl Environ Microbiol 64(11):4346–4352
24. Choi SH, Gu MB (2002) A portable toxicity biosensor using freeze-dried recombinant bioluminescent bacteria. Biosens Bioelectron 17(5):433–440
25. Dardenne F, Smolders R, De Coen W, Blust R (2007) Prokaryotic gene profiling assays to detect sediment toxicity: evaluating the ecotoxicological relevance of a cell-based assay. Environ Sci Technol 41(5):1790–1796
26. Elad T, Benovich E, Magrisso S, Belkin S (2008) Toxicant identification by a luminescent bacterial bioreporter panel: application of pattern classification algorithms. Environ Sci Technol 42(22):8486–8491
27. Pedahzur R, Polyak B, Marks RS, Belkin S (2004) Water toxicity detection by a panel of stress-responsive luminescent bacteria. J Appl Toxicol 24(5):343–348
28. Sazykin IS, Sazykina MA, Khmelevtsova LE, Mirina EA, Kudeevskaya EM, Rogulin EA, Rakin AV (2016) Biosensor-based comparison of the ecotoxicological contamination of the wastewaters of Southern Russia and Southern Germany. Int J Environ Sci Technol 13 (3):945–954
29. Hug C, Zhang X, Guan M, Krauss M, Bloch R, Schulze T, Reinecke T, Hollert H, Brack W (2015) Microbial reporter gene assay as a diagnostic and early warning tool for the detection and characterization of toxic pollution in surface waters. Environ Toxicol Chem 34:2523–2532
30. Elad T, Belkin S (2013) Broad spectrum detection and "barcoding" of water pollutants by a genome-wide bacterial sensor array. Water Res 47(11):3782–3790
31. Van Dyk TK, DeRose EJ, Gonye GE (2001) LuxArray, a high-density, genomewide transcription analysis of *Escherichia coli* using bioluminescent reporter strains. J Bacteriol 183 (19):5496–5505
32. Zaslaver A, Bren A, Ronen M, Itzkovitz S, Kikoin I, Shavit S, Liebermeister W, Surette MG, Alon U (2006) A comprehensive library of fluorescent transcriptional reporters for *Escherichia coli*. Nat Methods 3(8):623–628
33. Onnis-Hayden A, Weng H, He M, Hansen S, Ilyin V, Lewis K, Gu AZ (2009) Prokaryotic real-time gene expression profiling for toxicity assessment. Environ Sci Technol 43 (12):4574–4581

34. Gou N, Onnis-Hayden A, Gu AZ (2010) Mechanistic toxicity assessment of nanomaterials by whole-cell-array stress genes expression analysis. Environ Sci Technol 44(15):5964–5970
35. Zhang X, Wiseman S, Yu H, Liu H, Giesy JP, Hecker M (2011) Assessing the toxicity of naphthenic acids using a microbial genome wide live cell reporter array system. Environ Sci Technol 45(4):1984–1991
36. Su G, Zhang X, Raine JC, Xing L, Higley E, Hecker M, Giesy JP, Yu H (2013) Mechanisms of toxicity of triphenyltin chloride (TPTC) determined by a live cell reporter array. Environ Sci Pollut Res Int 20(2):803–811
37. Su G, Yu H, Lam MHW, Giesy JP, Zhang X (2014) Mechanisms of toxicity of hydroxylated polybrominated diphenyl ethers (HO-PBDEs) determined by toxicogenomic analysis with a live cell array coupled with mutagenesis in *Escherichia coli*. Environ Sci Technol 48 (10):5929–5937
38. Gou N, Yuan S, Lan J, Gao C, Alshawabkeh AN, Gu AZ (2014) A quantitative toxicogenomics assay reveals the evolution and nature of toxicity during the transformation of environmental pollutants. Environ Sci Technol 48(15):8855–8863
39. Guan M, Su G, Giesy JP, Zhang X (2016) Classification and toxicity mechanisms of novel flame retardants (NFRs) based on whole genome expression profiling. Chemosphere 144:2150–2157
40. Krivoshiev BV, Dardenne F, Blust R, Covaci A, Husson SJ (2015) Elucidating toxicological mechanisms of current flame retardants using a bacterial gene profiling assay. Toxicol Vitro 29(8):2124–2132
41. Gao C, Weisman D, Lan J, Gou N, Gu AZ (2015) Toxicity mechanisms identification via gene set enrichment analysis of time-series toxicogenomics data: impact of time and concentration. Environ Sci Technol 49(7):4618–4626
42. Janion C (2008) Inducible SOS response system of DNA repair and mutagenesis in *Escherichia coli*. Int J Biol Sci 4(6):338–344
43. Little JW, Edmiston SH, Pacelli LZ, Mount DW (1980) Cleavage of the *Escherichia coli lexA* protein by the *recA* protease. Proc Natl Acad Sci U S A 77(6):3225–3229
44. Quillardet P, Huisman O, D'Ari R, Hofnung M (1982) SOS chromotest, a direct assay of induction of an SOS function in *Escherichia coli* K-12 to measure genotoxicity. Proc Natl Acad Sci U S A 79(19):5971–5975
45. Oda Y, Nakamura S, Oki I, Kato T, Shinagawa H (1985) Evaluation of the new system (*umu* test) for the detection of environmental mutagens and carcinogens. Mutat Res 147 (5):219–229
46. Quillardet P, Hofnung M (1993) The SOS chromotest: a review. Mutat Res 297(3):235–279
47. Reifferscheid G, Heil J (1996) Validation of the SOS/*umu* test using test results of 486 chemicals and comparison with the Ames test and carcinogenicity data. Mutat Res 369:129–145
48. ISO (2000) ISO 13829:2000 Water quality – determination of the genotoxicity of water and waste water using the *umu*-test. ISO, Geneva
49. van der Lelie D, Regniers L, Borremans B, Provoost A, Verschaeve L (1997) The VITOTOX® test, an SOS bioluminescence *Salmonella typhimurium* test to measure genotoxicity kinetics. Mutat Res 389(2–3):279–290
50. Verschaeve L (2002) Genotoxicity studies in groundwater, surface waters, and contaminated soil. ScientificWorldJournal 2:1247–1253
51. Verschaeve L, Van Gompel J, Thilemans L, Regniers L, Vanparys P, van der Lelie D (1999) VITOTOX® bacterial genotoxicity and toxicity test for the rapid screening of chemicals. Environ Mol Mutagen 33(3):240–248
52. Westerink WMA, Stevenson JCR, Lauwers A, Griffioen G, Horbach GJ, Schoonen WGEJ (2009) Evaluation of the Vitotox™ and RadarScreen assays for the rapid assessment of genotoxicity in the early research phase of drug development. Mutat Res 676(1–2):113–130
53. Nunoshiba T, Nishioka H (1991) 'Rec-lac test' for detecting SOS-inducing activity of environmental genotoxic substances. Mutat Res 254(1):71–77

54. Vollmer AC, Belkin S, Smulski DR, Van Dyk TK, LaRossa RA (1997) Detection of DNA damage by use of *Escherichia coli* carrying *recA′::lux*, *uvrA′::lux*, or *alkA′::lux* reporter plasmids. Appl Environ Microbiol 63(7):2566–2571
55. Jia K, Marks RS, Ionescu RE (2014) Influence of carbon-based nanomaterials on *lux*-bioreporter *Escherichia coli*. Talanta 126:208–213
56. Song Y, Jiang B, Tian S, Tang H, Liu Z, Li C, Jia J, Huang WE, Zhang X, Li G (2014) A whole-cell bioreporter approach for the genotoxicity assessment of bioavailability of toxic compounds in contaminated soil in China. Environ Pollut 195:178–184
57. Frey J, Ghersa P, Palacios PG, Belet M (1986) Physical and genetic analysis of the ColD plasmid. J Bacteriol 166(1):15–19
58. Ptitsyn LR, Horneck G, Komova O, Kozubek S, Krasavin EA, Bonev M, Rettberg P (1997) A biosensor for environmental genotoxin screening based on an SOS *lux* assay in recombinant *Escherichia coli* cells. Appl Environ Microbiol 63(11):4377–4384
59. Østergaard TG, Hansen LH, Binderup M-L, Norman A, Sørensen SJ (2007) The *cda* GenoTox assay: a new and sensitive method for detection of environmental genotoxins, including nitroarenes and aromatic amines. Mutat Res 631(2):77–84
60. Busam S, McNabb M, Wackwitz A, Senevirathna W, Beggah S, van der Meer JR, Wells M, Breuer U, Harms H (2007) Artificial neural network study of whole-cell bacterial bioreporter response determined using fluorescence flow cytometry. Anal Chem 79(23):9107–9114
61. Norman A, Hansen LH, Sørensen SJ (2006) A flow cytometry-optimized assay using an SOS–green fluorescent protein (SOS–GFP) whole-cell biosensor for the detection of genotoxins in complex environments. Mutat Res 603(2):164–172
62. Biran A, Yagur-Kroll S, Pedahzur R, Buchinger S, Reifferscheid G, Ben-Yoav H, Shacham-Diamand Y, Belkin S (2010) Bacterial genotoxicity bioreporters. Microb Biotechnol 3:412–427
63. Volkert MR (1988) Adaptive response of *Escherichia coli* to alkylation damage. Environ Mol Mutagen 11(2):241–255
64. Hwang ET, Ahn J-M, Kim BC, Gu MB (2008) Construction of a *nrdA::luxCDABE* fusion and its use in *Escherichia coli* as a DNA damage biosensor. Sensors 8(2):1297–1307
65. Ahn J-M, Gu MB (2012) Geno-Tox: cell array biochip for genotoxicity monitoring and classification. Appl Biochem Biotechnol 168(4):752–760
66. Ahn J-M, Hwang ET, Youn C-H, Banu DL, Kim BC, Niazi JH, Gu MB (2009) Prediction and classification of the modes of genotoxic actions using bacterial biosensors specific for DNA damages. Biosens Bioelectron 25(4):767–772
67. Purvis IJ, Chotai D, Dykes CW, Lubahn DB, French FS, Wilson EM, Hobden AN (1991) An androgen-inducible expression system for *Saccharomyces cerevisiae*. Gene 106(1):35–42
68. Routledge EJ, Sumpter JP (1996) Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environ Toxicol Chem 15 (3):241–248
69. Raman DR, Williams EL, Layton AC, Burns RT, Easter JP, Daugherty AS, Mullen MD, Sayler GS (2004) Estrogen content of dairy and swine wastes. Environ Sci Technol 38 (13):3567–3573
70. Conroy O, Quanrud DM, Ela WP, Wicke D, Lansey KE, Arnold RG (2005) Fate of wastewater effluent hER-agonists and hER-antagonists during soil aquifer treatment. Environ Sci Technol 39(7):2287–2293
71. Buchinger S, Spira D, Bröder K, Schlüsener M, Ternes T, Reifferscheid G (2013) Direct coupling of thin-layer chromatography with a bioassay for the detection of estrogenic compounds: applications for effect-directed analysis. Anal Chem 85(15):7248–7256
72. Bovee TFH, Helsdingen RJR, Koks PD, Kuiper HA, Hoogenboom RLAP, Keijer J (2004) Development of a rapid yeast estrogen bioassay, based on the expression of green fluorescent protein. Gene 325:187–200

73. Bovee TFH, Helsdingen RJR, Hamers ARM, van Duursen MBM, Nielen MWF, Hoogenboom RLAP (2007) A new highly specific and robust yeast androgen bioassay for the detection of agonists and antagonists. Anal Bioanal Chem 389(5):1549–1558
74. Michelini E, Leskinen P, Virta M, Karp M, Roda A (2005) A new recombinant cell-based bioluminescent assay for sensitive androgen-like compound detection. Biosens Bioelectron 20(11):2261–2267
75. Sanseverino J, Eldridge ML, Layton AC, Easter JP, Yarbrough J, Schultz TW, Sayler GS (2009) Screening of potentially hormonally active chemicals using bioluminescent yeast bioreporters. Toxicol Sci 107(1):122–134
76. Wang J, Eldridge M, Menn FM, Dykes T, Sayler G (2015) Standardized application of yeast bioluminescent reporters as endocrine disruptor screen for comparative analysis of wastewater effluents from membrane bioreactor and traditional activated sludge. Ecotoxicology 24 (10):2088–2099
77. OECD (2015) Test no. 455: performance-based test guideline for stably transfected transactivation in vitro assays to detect estrogen receptor agonists and antagonists, OECD Guidelines for the Testing of Chemicals, Section 4. OECD, Paris
78. van der Burg B, van der Linden S, Man H-y, Winter R, Jonker L, van Vugt-Lussenburg B, Brouwer A (2013) A panel of quantitative Calux® reporter gene assays for reliable high-throughput toxicity screening of chemicals and complex mixtures. In: Steinberg P (ed) High-throughput screening methods in toxicity testing. Wiley, Hoboken, pp 519–532
79. White SS, Birnbaum LS (2009) An overview of the effects of dioxins and dioxin-like compounds on vertebrates, as documented in human and ecological epidemiology. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 27(4):197–211
80. Aarts JMMJG, Denison MS, De Haan LHJ, Schalk JAC, Cox MA, Brouwer A (1993) Ah receptor-mediated luciferase expression: a tool for monitoring dioxin-like toxicity. Organohalogen Compd 13:361–364
81. Denison MS, El-Fouly MH, Aarts JMMJG, Brouwer A, Richter C, Giesy JP (1993) Production of novel recombinant cell line bioassay systems for detection of 2,3,7,8-tetrachlorodibenzo-p-dioxin-like chemicals. Organohalogen Compd 13:365–368
82. Hoogenboom R, Bovee T, Traag W, Hoogerbrugge R, Baumann B, Portier L, van de Weg G, de Vries J (2006) The use of the DR CALUX bioassay and indicator polychlorinated biphenyls for screening of elevated levels of dioxins and dioxin-like polychlorinated biphenyls in eel. Mol Nutr Food Res 50(10):945–957
83. Besselink H, Leonards P, Felzel E, Brouwer B (2002) Analysis of polychlorinated dibenzo-p-dioxins (PCDD), dibenzofurans (PCDF) and biphenyls (PCB) in fish using DR CALUX® and GC/MS: a comparison. Organohalogen Compd 58:412–415
84. Besselink HT, Schipper C, Klamer H, Leonards P, Verhaar H, Felzel E, Murk AJ, Thain J, Hosoe K, Schoeters G, Legler J, Brouwer B (2004) Intra- and interlaboratory calibration of the DR CALUX® bioassay for the analysis of dioxins and dioxin-like chemicals in sediments. Environ Toxicol Chem 23:2781–2789
85. Whyte JJ, Schmitt CJ, Tillitt DE (2004) The H4IIE cell bioassay as an indicator of dioxin-like chemicals in wildlife and the environment. Crit Rev Toxicol 34(1):1–83
86. Petrulis JR, Bunce NJ (1999) Competitive inhibition by inducer as a confounding factor in the use of the ethoxyresorufin-O-deethylase (EROD) assay to estimate exposure to dioxin-like compounds. Toxicol Lett 105(3):251–260
87. Denison MS, Zhao B, Baston DS, Clark GC, Murata H, Han D (2004) Recombinant cell bioassay systems for the detection and relative quantitation of halogenated dioxins and related chemicals. Talanta 63(5):1123–1133
88. Turner K, Xu S, Pasini P, Deo S, Bachas L, Daunert S (2007) Hydroxylated polychlorinated biphenyl detection based on a genetically engineered bioluminescent whole-cell sensing system. Anal Chem 79(15):5740–5745
89. Kramer KJM, Foekema EM (2000) The "Musselmonitor®" as a biological early warning system. In: Butterworth FM, Gunatilaka A, Gonsebatt ME (eds) Biomonitors and biomarkers

as indicators of environmental change 2. Kluwer Academic/Plenum Publishers, New York, pp 59–88
90. van der Schalie WH, Shedd TR, Knechtges PL, Widder MW (2001) Using higher organisms in biological early warning systems for real-time toxicity detection. Biosens Bioelectron 16:457–465
91. Gerhardt A, Janssens de Bisthoven L, Penders E (2003) Quality control of drinking water from the River Rhine (Netherlands) with the multispecies freshwater biomonitor. Aquat Ecosyst Health Manag 6(2):159–166
92. Gunatilaka A, Diehl P, Puzicha H (2000) The evaluation of the "Dynamic Daphnia Test" after a decade of use. In: Butterworth FM, Gunatilaka A, Gonsebatt ME (eds) Biomonitors and biomarkers as indicators of environmental change 2. Kluwer Academic/Plenum Publishers, New York, pp 29–58
93. Gerhardt A, Ingram MK, Kang IJ, Ulitzur S (2006) In situ on-line toxicity biomonitoring in water: recent developments. Environ Toxicol Chem 25(9):2263–2271
94. Kim BC, Gu MB (2005) A multi-channel continuous water toxicity monitoring system: its evaluation and application to water discharged from a power plant. Environ Monit Assess 109:123–133
95. Lee JH, Mitchell RJ, Kim BC, Cullen DC, Gu MB (2005) A cell array biosensor for environmental toxicity analysis. Biosens Bioelectron 21(3):500–507
96. Bjerketorp J, Håkansson S, Belkin S, Jansson JK (2006) Advances in preservation methods: keeping biosensor microorganisms alive and active. Curr Opin Biotechnol 17(1):43–49
97. Heitzer A, Malachowsky K, Thonnard JE, Bienkowski PR, White DC, Sayler GS (1994) Optical biosensor for environmental on-line monitoring of naphthalene and salicylate bioavailability with an immobilized bioluminescent catabolic reporter bacterium. Appl Environ Microbiol 60:1487–1494
98. Eltzov E, Marks RS, Voost S, Wullings BA, Heringa MB (2009) Flow-through real time bacterial biosensor for toxic compounds in water. Sens Actuat B Chem 142:11–18
99. Eltzov E, Slobodnik V, Ionescu RE, Marks RS (2015) On-line biosensor for the detection of putative toxicity in water contaminants. Talanta 132:583–590
100. Charrier T, Chapeau C, Bendria L, Picart P, Daniel P, Thouand G (2011) A multichannel bioluminescent bacterial biosensor for the on-line detection of metals and toxicity. Part II: technical development and proof of concept of the biosensor. Anal Bioanal Chem 400:1061–1070
101. Elad T, Almog R, Yagur-Kroll S, Levkov K, Melamed S, Shacham-Diamand Y, Belkin S (2011) Online monitoring of water toxicity by use of bioluminescent reporter bacterial biochips. Environ Sci Technol 45(19):8536–8544
102. Nivens DE, McKnight TE, Moser SA, Osbourn SJ, Simpson ML, Sayler GS (2004) Bioluminescent bioreporter integrated circuits: potentially small, rugged and inexpensive whole-cell biosensors for remote environmental monitoring. J Appl Microbiol 96(1):33–46
103. Vijayaraghavan R, Islam SK, Zhang M, Ripp S, Caylor S, Bull ND, Moser S, Terry SC, Blalock BJ, Sayler GS (2007) A bioreporter bioluminescent integrated circuit for very low-level chemical sensing in both gas and liquid environments. Sens Actuat B Chem 123 (2):922–928
104. Eltzov E, Yehuda A, Marks RS (2015) Creation of a new portable biosensor for water toxicity determination. Sens Actuat B Chem 221:1044–1054
105. Eltzov E, Cohen A, Marks RS (2015) Bioluminescent liquid light guide pad biosensor for indoor air toxicity monitoring. Anal Chem 87(7):3655–3661
106. Stocker J, Balluch D, Gsell M, Harms H, Feliciano J, Daunert S, Malik KA, van der Meer JR (2003) Development of a set of simple bacterial biosensors for quantitative and rapid measurements of arsenite and arsenate in potable water. Environ Sci Technol 37 (20):4743–4750

107. Struss A, Pasini P, Ensor CM, Raut N, Daunert S (2010) Paper strip whole cell biosensors: a portable test for the semiquantitative detection of bacterial quorum signaling molecules. Anal Chem 82(11):4457–4463
108. Date A, Pasini P, Daunert S (2010) Integration of spore-based genetically engineered whole-cell sensing systems into portable centrifugal microfluidic platforms. Anal Bioanal Chem 398 (1):349–356
109. Date A, Pasini P, Sangal A, Daunert S (2010) Packaging sensing cells in spores for long-term preservation of sensors: a tool for biomedical and environmental analysis. Anal Chem 82 (14):6098–6103
110. Sangal A, Pasini P, Daunert S (2011) Stability of spore-based biosensing systems under extreme conditions. Sens Actuat Chem 158(1):377–382
111. Gilmore J, Islam M, Martinez-Duarte R (2016) Challenges in the use of compact disc-based centrifugal microfluidics for healthcare diagnostics at the extreme point of care. Micromachines 7(4):52
112. Roda A, Cevenini L, Michelini E, Branchini BR (2011) A portable bioluminescence engineered cell-based biosensor for on-site applications. Biosens Bioelectron 26 (8):3647–3653
113. Cevenini L, Calabretta MM, Tarantino G, Michelini E, Roda A (2016) Smartphone-interfaced 3D printed toxicity biosensor integrating bioluminescent "sentinel cells". Sens Actuat B Chem 225:249–257
114. Biran A, Ben Yoav H, Yagur-Kroll S, Pedahzur R, Buchinger S, Shacham-Diamand Y, Reifferscheid G, Belkin S (2011) Microbial genotoxicity bioreporters based on *sulA* activation. Anal Bioanal Chem 400(9):3013–3024
115. Ben-Yoav H, Biran A, Pedahzur R, Belkin S, Buchinger S, Reifferscheid G, Shacham-Diamand Y (2009) A whole cell electrochemical biosensor for water genotoxicity bio-detection. Electrochim Acta 54(25):6113–6118
116. Tsai H-F, Tsai Y-C, Yagur-Kroll S, Palevsky N, Belkin S, Cheng J-Y (2015) Water pollutant monitoring by a whole cell array through lens-free detection on CCD. Lab Chip 15 (6):1472–1480
117. Siegfried K, Endes C, Bhuiyan AF, Kuppardt A, Mattusch J, van der Meer JR, Chatzinotas A, Harms H (2012) Field testing of arsenic in groundwater samples of Bangladesh using a test kit based on lyophilized bioreporter bacteria. Environ Sci Technol 46(6):3281–3287
118. Siegfried K, Hahn-Tomer S, Koelsch A, Osterwalder E, Mattusch J, Staerk H-J, Meichtry JM, De Seta GE, Reina FD, Panigatti C, Litter MI, Harms H (2015) Introducing simple detection of bioavailable arsenic at Rafaela (Santa Fe Province, Argentina) using the ARSOlux biosensor. Int J Environ Res Public Health 12(5):5465–5482
119. Baumstark-Khan C, Rode A, Rettberg P, Horneck G (2001) Application of the Lux-Fluoro test as bioassay for combined genotoxicity and cytotoxicity measurements by means of recombinant *Salmonella typhimurium* TA1535 cells. Anal Chim Acta 437(1):23–30
120. Rettberg P, Bandel K, Baumstark-Khan C, Horneck G (2001) Increased sensitivity of the SOS-LUX-Test for the detection of hydrophobic genotoxic substances with *Salmonella typhimurium* TA1535 as host strain. Anal Chim Acta 426(2):167–173
121. Davidov Y, Rozen R, Smulski DR, Van Dyk TK, Vollmer AC, Elsemore DA, LaRossa RA, Belkin S (2000) Improved bacterial SOS promoter::*lux* fusions for genotoxicity detection. Mutat Res 466(1):97–107
122. Maehana K, Tani H, Shiba T, Kamidate T (2004) Effects of using a low-copy plasmid and controlling membrane permeability in SOS-based genotoxic bioassay. Anal Chim Acta 522 (2):189–195
123. Norman A, Hestbjerg Hansen L, Sørensen SJ (2005) Construction of a ColD cda promoter-based SOS-green fluorescent protein whole-cell biosensor with higher sensitivity toward genotoxic compounds than constructs based on *recA*, *umuDC*, or *sulA* promoters. Appl Environ Microbiol 71(5):2338–2346

124. Dreier J, Breitmaier EB, Gocke E, Apfel CM, Page MGP (2002) Direct influence of S9 liver homogenate on fluorescence signals: impact on practical applications in a bacterial genotoxicity assay. Mutat Res 513(1–2):169–182
125. Arai R, Makita Y, Oda Y, Nagamune T (2001) Construction of green fluorescent protein reporter genes for genotoxicity test (SOS/*umu*-test) and improvement of mutagen-sensitivity. J Biosci Bioeng 92(3):301–304
126. Yagur-Kroll S, Bilic B, Belkin S (2010) Strategies for enhancing bioluminescent bacterial sensor performance by promoter region manipulation. Microb Biotechnol 3:300–310
127. Yagur-Kroll S, Belkin S (2011) Upgrading bioluminescent bacterial bioreporter performance by splitting the *lux* operon. Anal Bioanal Chem 400(4):1071–1082
128. Allen MS, Wilgus JR, Chewning CS, Sayler GS, Simpson ML (2007) A destabilized bacterial luciferase for dynamic gene expression studies. Syst Synth Biol 1(1):3–9
129. Malling HV (1971) Dimethylnitrosamine: formation of mutagenic compounds by interaction with mouse liver microsomes. Mutat Res 13(4):425–429
130. Oda Y, Shimada T, Watanabe M, Ishidate M, Nohmi T (1992) A sensitive umu test system for the detection of mutagenic nitroarenes in *Salmonella typhimurium* NM1011 having a high nitroreductase activity. Mutat Res 272(2):91–99
131. Oda Y, Yamazaki H, Watanabe M, Nohmi T, Shimada T (1993) Highly sensitive *umu* test system for the detection of mutagenic nitroarenes in *Salmonella typhimurium* NM3009 having high O-acetyltransferase and nitroreductase activities. Environ Mol Mutagen 21 (4):357–364
132. Oda Y, Yamazaki H, Watanabe M, Nohmi T, Shimada T (1995) Development of high sensitive *umu* test system: rapid detection of genotoxicity of promutagenic aromatic amines by *Salmonella typhimurium* strain NM2009 possessing high O-acetyltransferase activity. Mutat Res 334(2):145–156
133. Oda Y, Kunihiro F, Masaaki K, Akihiko N, Taro Y (2004) Use of a high-throughput *umu*-microplate test system for rapid detection of genotoxicity produced by mutagenic carcinogens and airborne particulate matter. Environ Mol Mutagen 43(1):10–19