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REVIEW

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Reporter gene bioassays in environmental analysis

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Abstract In parallel to the continuous development of increasingly more sophisticated physical and chemical analytical technologies for the detection of environmental pollutants, there is a progressively more urgent need also for bioassays which report not only on the presence of a chemical but also on its bioavailability and its biological effects. As a partial fulfillment of that need, there has been a rapid development of biosensors based on genetically engineered bacteria. Such microorganisms typically combine a promoter-operator, which acts as the sensing element, with reporter gene(s) coding for easily detectable proteins. These sensors have the ability to detect global parameters such as stress conditions, toxicity or DNA-damaging agents as well as specific organic and inorganic compounds. The systems described in this review, designed to detect different groups of target chemicals, vary greatly in their detection limits, specificity, response times and more. These variations reflect on their potential applicability which, for most of the constructs described, is presently rather limited. Nevertheless, present trends promise that additional improvements will make microbial biosensors an important tool for future environmental analysis.

Introduction

The increasing awareness of the environmental problems caused by industrial and agricultural pollution has created a demand for progressively more sophisticated detection methods. In response, many chemical and physical methodologies were developed along with the required analytical equipment: gas or high pressure liquid chromatography, mass and atomic absorption spectrometry, etc. The resulting techniques – powerful, accurate and sensitive – are also costly and require specialized laboratories. In addition, they fail to provide data as to the bioavailability of a pollutant, its effects on living systems, or its potential synergistic/antagonistic behavior in mixtures.

Thus, in parallel to the advances in the analytical approaches, an increasingly varied set of bioassays has also been under continuous development for environmental monitoring purposes. A variety of organisms, cellular or subcellular systems has been employed for these purposes, from whole-organism assays such as fish toxicity testing to immunological determination of specific pesticides. Among the test organisms, a special position is held by bacteria: their large population sizes, rapid growth rates, low costs and easy maintenance often make them a more attractive option than other systems. Furthermore, as is becoming increasingly obvious, bacteria are endowed with an additional characteristic which further augments their attractiveness: they are readily amenable to genetic manipulation. Thus, by relatively simple molecular biology techniques, bacterial strains can be "tailored" to emit a detectable signal upon a pre-specified change in environmental conditions. As will be discussed below, several sets of reporter proteins have been used for this purpose. The present review attempts to summarize the status of the application of these reporter genes in recombinant microorganisms for environmental monitoring. Following the introduction of the general concept and the available reporting tools in the following two sections, this summary discusses recombinant bacterial sensors in two groups: strains designed to sense individual compounds (heavy metals or specific organics), and others which are tailored to report on "group parameters" such as toxicity or genotoxicity.

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General concept

In order for a bacterial cell to function as a "microbial bioreporter" or a "microbial biosensor", it has to contain two linked genetic elements: a sensing element and a reporter. The former senses the presence of the target molecule(s), and turns on the latter which emits a detectable signal. The reporter element is always one of a typical set of genes or groups of genes, coding for proteins with an easily detectable presence or activity, as will be described in more detail below.

The sensing element, in contrast, is different in each bacterial sensor, and in its selection lies the uniqueness and specificity of the final construct. In most cases, the sensing element is a promoter for a gene or a group of genes normally activated in response to a specific or general environmental change. Under normal conditions, this activation would lead to the synthesis of proteins, the presence or activity of which would help the cell combat the sensed hazard or adapt to it. In the recombinant strain, in addition to this function, the selected promoter also drives the synthesis of the reporter protein(s).

Using this principle, a promoter sequence from one bacterial species can be genetically fused to a reporter gene from a second species and introduced into the cells of a third microorganism. In practice, in order for the promoter sequence to sense its target chemicals, an additional element has often to be included: the regulatory mechanism mediating the signal from the sensed molecule to the promoter. This can easily be achieved if the host cell is also the origin of the promoter used.

The fused promoter::reporter can be introduced into the host cell in one of two options: either as a plasmid, normally a multicopy one, or integrated into the bacterial chromosome, as depicted in Fig. 1. The latter option, which calls for a somewhat lengthier molecular procedure, allows better stability of the system but may suffer from a reduced signal strength.

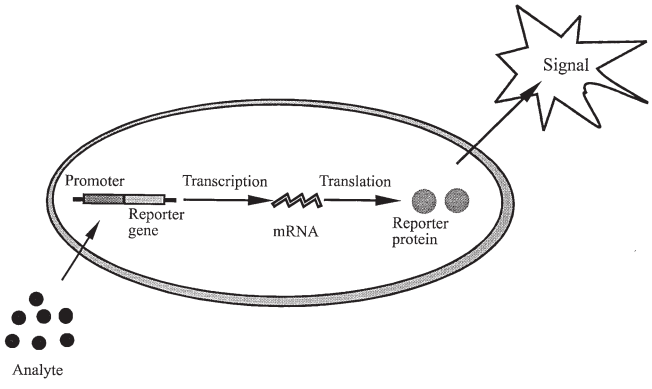

Fig. 1 Theory of the measurement (after [1])

Reporter genes and proteins

The list of different regulatory proteins used for recombinant environmental sensing outlined in Table 1 is similar to those used in other gene expression assays. All the reporters on that list are either readily detectable (GFP) or are capable of an easily measured activity. Just as the specificity of the final construct depends upon the proper selection of the sensing promoter, the facility, sensitivity and degree of resolution of the detection will depend to a large extent upon the proper choice of the reporter.

Bacterial **β-galactosidase** catalyzes the hydrolysis of β-galactosides. This enzyme is encoded by *lacZ* of *Escherichia coli* and can be used in prokaryotic as well as in eukaryotic cells. The enzyme has a high turnover rate and generates strong signals by using fluorescence, electrochemical or chemiluminescence substrates [1]. The enzyme can be measured with a sensitivity of less than 1 amol, depending upon the substrate used [2].

Bacterial **luciferases** catalyze the obligately aerobic oxidation of a reduced flavin mononucleotide and a long chain aldehyde to flavin mononucleotide and the corresponding carboxylic acid, with light emission at around 490 nm and a quantum yield of ~ 0.1. The luciferase is encoded by *luxA* and *luxB* of the *lux* operon, and the synthesis enzymes for the aldehyde are coded by *luxCDE* [3]. In constructs where only *luxAB* is present, the aldehyde has to be added externally. The commonly used luciferases of *Vibrio fischeri* and *Vibrio harveyi* have a limited temperature range < 30 °C or < 37 °C, respectively. In some applications *Photorhabdus (Xenorhabdus) luminescens* is used due to its higher upper temperature limit (45 °C) [4].

Table 1 Reporter genes and proteins

Reporter protein	Reporter gene	Origin	Potential substrate	Detection method	References
Bacterial luciferase	*lux*	One of several luminescent bacteria	Long chain aldehydes (C9–C14)	Luminescence	[83]
Insect luciferase	*luc*	Fireflies, click beetles	Luciferin	Luminescence	[83]
β-Galactosidase	*lacZ*	*E. coli*	Galactopyranosides	Colorimetric, electrochemical, fluorescence, chemiluminescence	[84, 85]
Green fluorescent protein	*gfp*	*Aequorea victoria*	–	Fluorescence	[7]
Alkaline phosphatase	*phoA*	Various	Phosporylated organics	Colorimetric, chemiluminescence	[9, 86]
β-Glucuronidase	*uidA (gusA, gurA)*	*E. coli*	β-Glucuronides	Colorimetric, fluorescence, luminescence	[9]
β-Lactamase	*bla*	*E.coli*	Lactamides	Colorimetric	[10, 17, 85]

The firefly **luciferase** is encoded by the *luc* gene. The oxygen-dependent bioluminescent reaction is based on energy transfer from ATP to the substrate, D-luciferin, to yield oxyluciferin, AMP, carbon dioxide and light (560 nm) [5]. The quantum yield of 0.88 is the highest known for bioluminescent reactions [6]. The substrate, luciferin, has always to be added externally.

GFP, **green fluorescent protein**, isolated from the jellyfish *Aequorea victoria*, fluoresces in this animal due to an energy transfer from the Ca^{2+}-activated photoprotein aequorin [7]. The highly stable GFP protein has a high quantum yield (0.88), and can be expressed in both prokaryotic and eukaryotic systems with no need of a substrate or cofactor [7]. GFP does not have any enzymatic amplification, thus reducing its potential detection sensitivity. It has been shown that 10^5 to 10^6 GFP molecules are needed to allow detection over background fluorescence in a single cell [8].

β-glucuronidase (GUS) is mainly used for plant cells and there are some reports of its application in mammalian systems. Different substrates are known, forming colored, fluorescent, or luminescent cleavage products; the sensitivity of the luminescent assay is the highest [9].

Alkaline phosphatase (AP) and secreted alkaline phosphatase (SEAP) are orthophosphoric monoester phosphohydrolases with an alkaline pH optimum. These stable enzymes are characterized by a high turnover rate. The classical determination is colorimetric, but extremely sensitive fluorescent and luminometric detection systems were reported; the latter allow a sensitivity similar or better compared to that of luciferase (0.05–0.01 amol) [2].

Some bacteria can be penicillin-resistant by producing **β-lactamase**. β-lactamase is the enzyme that cleaves β-lactam rings in antibiotics, such as penicillin. The use of substrates cleaving fluorescent or colorimetric products allow the detection of this enzyme [8, 10].

While some of the bioluminescent methods were recently reviewed by Steinberg et al. [11], a comprehensive study of available "environmental" recombinant constructs was not available. This review attempts to partially answer this need by reviewing examples of the uses of these reporters in a variety of genetic constructs.

Individual inorganic and organic compounds

Heavy metals

Cadmium, arsenic

Many of the environmental reporter gene assays are devoted to the detection of heavy metals (Table 2). In most cases, promoters of genes involved in metal-resistance mechanisms are used as the sensing elements. A short overview of the monitoring of toxic metals, with an emphasis on arsenic and mercury, was published by Ramanathan et al. [12].

Alcaligenes eutrophus, which harbors two megaplasmids (pMOL28 and pMOL30) governing multiple resistance to heavy metals, served as a source for promoters used in several bioassays [13], as was plasmid pI258 of *Staphylococcus aureus* [14, 15]. Corbisier et al. [14] described a shuttle vector between *E. coli* and *S. aureus* containing *arsB::luxAB* or *cadA::luxAB*. Arsenite was a stronger inducer in *E. coli* HB101 with a maximum light emission at 10 μM, followed by arsenate and bismuth. In *S. aureus* the system showed a pronounced specificity to arsenite. The same effect was also obtained using a different reporter gene, *blaZ*, in *E. coli* HB101 as host [16]. The most effective strain for the induction with Cd^{2+} was *S. aureus* RN4220 with the *cadA::luxAB* fusion [14]. Yoon et al. [17] constructed a translational *cadA-blaZ* fusion and reported β-lactamase activity when induced with Cd^{2+}, Bi^{3+} and Pb^{2+}. The Cd^{2+} concentration allowing maximal β-lactamase activity was ten times lower than that promoting maximum light emission.

The cloning of the *cadC* gene and the promoter/operator of the *cadA* operon from the plasmid pI258 in front of the *luc* gene created a sensor responsive to cadmium and lead [18]. In *Staphylococcus aureus* the detection limit of 10 nM was somewhat higher than the 3.3 nM in *Bacillus subtilis*. Freeze-drying affected the sensitivity and/or efficiency of the *S. aureus* and *Bacillus* constructs. The detection limit reported for this *luc* system was better than that of other heavy metal bioreporters. This sensor strain also detected lead, antimony and tin.

Another detection method for arsenite or antimonite was developed by the fusion of the promoter of the *ars* operon to *lacZ*, in a plasmid which also contained *arsR*, the regulatory gene of this operon [19, 20]. The expressed β-galactosidase was detected by chemiluminescence [19] and electrochemistry [20] with permeabilized cells. The detection limit of the chemiluminescent assay was 10^{-15} M in 30 min for antimonite, while the electrochemical system could detect 10^{-7} M antimonite in 17 h.

Chromate

A highly specific bacterial chromate sensor reported by Peitzsch et al. [21] was based on *chr::lux* fusion, performing optimally with glycerol as a carbon source. Sulfate starvation repressed the induction by chromate, possibly by inducing the uptake and reduction of this ion by the sulfate reduction pathway.

Mercury

A variety of assays were developed for the detection of mercury [22, 23], with different promoters of the *mer* operon driving several reporter genes: *lux* [22–27] *luc* [28], *blaZ* [29], and *lacZ* [24, 30].

In the construct reported by Virta et al. [28] the firefly *luc* was under the control of *merR* of *Tn21*, with *E. coli* MC1061 as host, allowing a specific detection of mercury in the range of 0.1 fM to 0.1 μM. The sensitivity obtained here exceeded that in *lux* reporter bioassays described for

Table 2 Recombinant bacterial bioassay detection limits

Analyte	Promoter (origin)	Reporter	Micro-organism	Time for induction	Concentration	Reference
Inorganic compounds						
Aluminium	*fliC (E. coli)*	*luxAB (V. harveyi)*	*E. coli*	20 min	40–400 μM	[33]
Antimonite, arsenite	*arsRD′*	*lacZ*	*E. coli*	17 h 30 min	100 μM 10^{-15} M	[19, 20]
Arsenate, arsenite	*arsRDABC, arsRBC (E. coli, S. aureus)*	*luxAB (V. harveyi)*				[87]
	arsR	*luc (firefly)*	*S. aureus*	1 h	*ca.* 0.01–10 μM	[88]
Arsenic	*ars (S. aureus)*	*blaZ*	*S. aureus,E. coli*			[16]
	arsB (S. aureus)	*luxAB (V. harveyi)*	*S. aureus, E. coli*	1–2 h		[14]
Cadmium	*cadA (S. aureus)*	*luxAB (V. harveyi)*	*S. aureus, E. coli*	1–2 h	1–100 μM	[14]
	cadA, cadC (S. aureus)	*blaZ*	*S. aureus*	1.5 h	0.5–100 μM	[17]
	cadCo/p	*luc (firefly)*	*B. subtilis* *S. aureus*	3 h 2 h	10 nM 3.3 nM	[18]
	lac (E. coli)	*lux (V. fischeri)*		1 h	> 0.42 μM	[89]
Chromate	*chr (A. eutrophus)*	*lux*	*A. eutrophus*	1–2 h	1–50 nM	[21]
Copper		*luxABCDE (V. fisheri)*	*E. coli*		1 μM–1 mM	[54]
Heavy metals		*luxAB (V. harveyi)*				[90]
Iron	*pupA (P. putida)*	*luxCDABE (V. fischeri)*	*P. putida*	Hours	10^{-2}–1 μM	[37]
Inorganic mercury	*mer (Tn21)*	*luc (firefly)*	*E. coli*		≤ 0.1 fM	[28]
	merTPAP (TN501)	*lacZ, lux (V. fischeri)*	*E. coli*		0.02–0.2 μM	[24]
	mer (S. aureus)	*blaZ (S. aureus)*			5 μM	[29]
	mer (Tn21)	*luxAB (V. harveyi)*	*E. coli*	2–3 min	10^{-8} M	[22]
	mer (Tn21)	*luxCDABE (V. fischeri)*	*E. coli*	40 min	0.5–5 μM	[25]
	mer (Tn21)	*luxCDABE (V.o fischeri)*	*E. coli*		10 pM	[31]
	mer	*lux (V. fischeri)*	*E. coli*	30 min	10 nM–4 μM	[26]
	mer	*luxABCDE (V. fisheri)*	*E. coli*		0.1 nM–1 μM	[54]
	merR-T′	*luxCDABE (V. fischeri)*	*E. coli*	70 min	0.025 nM	[23]
	mer (S. marcesanans)	*lux (V. fischeri)*	*E. coli*	90 min		[27]
Metal salts, organic solvents	*lac*	*luxαβ (V. harveyi)*	*E. coli*	1 h	1–50 μM $CdCl_2$	[78]
Nitrate	*narG (E. coli)*	*luxCDABE (P. luminescens)*	*E. coli*	4 h	0.05–50 μM	[38]
Zinc	*smtA (Synechococcus PCC7942)*	*luxCDABE (V. fischeri)*	*Synechococcus PCC7942*	4 h	0.5–4 μM	[34]
	smtA (Synechococcus PCC7942)	*lacZ*	*Synechococcus PCC7942*	2 h	≤ 12 μM	[35]
Organic coumpounds						
Alkane	*alkB (P. oleovorans)*	*luxAB (V. harveyi)*	*E. coli*	15 min	24–100 nM	[51]
Benzene derivatives: m-xylene	*Ps of TOL (P. putida)*	*luc (firefly)*	*E. coli*	2 h	≤ 5 μM	[39]
	Ps of TOL (P. putida)	*luc (firefly)*	*Immob. E. coli*	1 h	0.05–1 mM	[41]
	xylR, Pu (P. putida)	*luc (firefly)*	*E. coli*	0.5–1 h	10–20 μM (toluene)	[43]
	xyl	*luxCDABE (V. fischeri)*	*P. putida*	Hours	3 μM (3-xylene)	[42]
4-Chlorobenzoate	*fcbA (Arthrobacter SU)*	*luxCDABE (V. fisheri)*	*E. coli*	1 h	380 μM–6.5 mM	[50]
Hydrocarbon pollution	*ibp (P. putida)*	*luxCDABE (V. fischeri)*	*E. coli*	1–4 h	1–100 μM (isopropyl-benzene) 0.1–10 μM (naphthalene)	[52]
Organic mercury	*merG (Tn501)*	*lacZ*	*E. coli*		0.3 nM	[30]
Naphthalene	*nahG (P. fluorescens)*	*luxCDABE (V. fischeri)*	*Immobilized P. fluorescens*	8–24 min	12–120 μM	[47]
Salicylate			*HK44*		36 uM	
PCB	*orf0-bphA1 (R. eutropha ENV307)*	*luxCDABE (V. fischeri)*	*R. eutropha*	1–3 h	0.8 μM (4-chlorobiphenyl) (Aroclor 1242)	[53]
Toluene	?	?	*P. putida*	?	0.1 μM	[44]

mercury by Selifonova et al. [25] and by Tescione and Belfort [26], in which the detection limit was ca. 0.1 nM. One possible explanation for the difference in the sensitivity could be ascribed to the different quantum yields of the firefly (90%) and bacterial (5%) systems. Selifonova et al. [25] proposed an increase in sensitivity by cloning transport systems in front of the reporter. Geiselhart et al. [27] cloned the *lux* genes under the control of *merR* in *E. coli*. Induction periods of 90 min were reported, but not the detection limit.

Klein et al. [30] expanded the system to detect organic mercury. The *merB* (organolyase) gene of *Streptomyces lividans* was cloned on a helper plasmid. This helper plasmid with *merTPB* and the reporter plasmid with *merRopTPlacZori* were cotransformed in *E. coli* JM109, leading to a detection threshold of 3×10^{-10} M phenylmercury acetate.

Rouch et al. [24] compared the detection of Co and Hg by the use of the *pcoE* and the *merTPAD* genes, respectively, with *lacZ* as the reporter. A gradual (hyposensitive) response for Co and a sharp (hypersensitive) threshold for Hg were observed with increasing concentrations of the test metals. A possible reason for this difference is that unlike Hg, Co is a micronutrient for the cells. Selifonova et al. [25] detected mercury with a limit of 1 nM by the use of the plasmid pRB28 containing a *merRo/pT′::luxCDABE* fusion. Sensitivity of the same construct was later improved to the picomolar range by Rasmussen et al. [31] by the use of low cell densities. An interesting calculation was introduced by Barkay et al. [23], attempting to express the bioavailability of Hg^{2+}, from the logarithmic increase in the rate of light production.

Aluminium

Bioassays for aluminium reported by Guzzo et al. [32, 33], using a chromosomal *fliC::luxAB* fusion, exhibited a pH dependence due to decreased solubility of aluminium at higher pH values. Dodecanal had to be added as substrate, both in a Petri dish assay [32] and in liquid culture [33]. Luminescence was induced in liquid media in only 20 min by the presence of 40–400 μM aluminium, but not by copper, iron or nickel [33]. In contrast, in the Petri dish assay a reaction was also induced by those three metals [32].

Zinc

Two different reporter genes were used for the detection of Zn, with the same operator-promoter: the *smt* gene from the cyanobacterium *Synechococcus* PCC7942. Erbe et al. [34] used *luxCDABE* from *Vibrio fischeri* and Huckle et al. [35] have selected *lacZ* as reporting element. The β-galactosidase assay required cell lysis prior to the determination of the enzyme activity [35], while the use of the *luxCDABE* reporter [34] required only the addition of dodecanal as a substrate, in spite of the presence of the *luxCDE* genes. Apparently, these genes did not produce sufficient substrate in the cyanobacterial cells to allow maximal activity. This assay responds, in addition to Zn^{2+}, also to Cu^{2+} and Cd^{2+}, but with longer induction times. The detection range for Zn was 0.5–4 μM, similar to that of atomic absorption spectroscopy [36] (Table 3).

Iron

An interesting variation, in which the absence of the compound is detected rather than its presence, was described by Khang et al. [37] for the determination of Fe. A fragment containing the *pupA* promoter of *Pseudomonas putida* WC358 was fused to the *luxCDABE* cassette of *Vibrio fischeri* and the bacterium was exposed to a strong chelator (2,2′-dipyridyl). In response to the applied iron starvation, bioluminescence increased with increasing chelator concentrations. The effect was neutralized by the addition of $FeCl_3$. Better detection was obtained in minimal (0.01–1 μM) than in complex media (0.1–10 mM).

Other inorganic compounds: nitrate

The only report of a genetically engineered bioassay for an inorganic compound other than a heavy metal was a nitrate sensor described by Prest et al. [38] in *E. coli* JM109. The *narG* promoter region for nitrate reductase was cloned in front of the *P. luminescens luxCDABE*. *NarG* is controlled by NarL which, after being phosphorylated by the nitrate sensor proteins NarX and NarQ, binds to the nitrate reductase promoter and activates its expression. This system could be used to detect nitrate in concentrations down to 0.5 μM. Nitrite and ammonium also induced the expression of luciferase, thus reducing the sensor's specificity; 2 h of incubation were required.

Organic compounds

To date, only a limited number of recombinant bioassays for the detection of organic pollutants were reported; these are listed in Table 2.

m-Xylene and benzene derivatives

The TOL plasmid of *Pseudomonas putida* contains the genes of the degradative enzymes for benzene and its derivatives, under the two-component regulatory system of *xylR* and *xylS*. The *xylR* protein forms a complex with the inducing aromatic compound, activating the *Ps* promoter of *xylS*. The *xylS* protein, in turn, activates the degradative activity. Kobatake et al. [39, 40] constructed plasmid pTSN316, which contained the luciferase gene of firefly, *luc* and *xylR* under the control of the promoter *Ps*. In *E. coli* HB10, following a 2 h lag period, m-xylene could be detected by this construct with a detection limit of 5 μM. Benzene derivatives such as toluene, ethylbenzene,

xylene, ethyltoluene and chlorotoluene also elicited a response, with the meta compounds being the more effective inducers. Possibly, the location of the side-chain affects the affinity of these compounds to the *xylR* protein [39]. As in all other uses of the *luc* reporter, luciferin had to be added as substrate. In order to facilitate entry of this hydrophobic molecule into the cells, they were either EDTA-treated or subjected to an acid treatment. In the latter case, highest bioluminescence was obtained at a pH of approximately 4.5.

EDTA-treated cells of the same construct were immobilized at the tip of an optic fiber with a base covered with either a dialysis or polycarbonate membrane. The dialysis membrane allowed a linear correlation between the m-xylene concentration and luminescence, but the required induction periods were longer than in non-immobilized cultures. Preincubation of the immobilized cells with m-methylbenzyl alcohol reduced the induction period to 1 h, and lowered the detection limit to 0.05–1 mM [41].

A different construct based on the *xyl* system was reported by Burlage [42], using *Pseudomonas* as a host instead of *E. coli* and the *V. fischeri lux* as a reporter. No detection limits were reported.

Another construct detecting benzene derivatives was reported by Willardson et al. [43], in which *luc* was placed under the control of *xylR* and *Pu*. *E. coli* cells transformed with this plasmid responded to toluene, xylene, and similar molecules. Detecton limit for toluene was 3.44 mM.

Simpson et al. [44] presented a prototype of a bioluminescent-bioreporter integrated circuit (BBIC) by placing the toluene-responsive bioreporter *Pseudomonas putida* TVA8 on an optical application-specific integrated circuit (OASIC). The OASIC is placed in a 40-pin ceramic chip carrier on an agar plug. Bioluminescence, induced by exposure to toluene vapors, is then measured by the photodiode of the BBIC. Long integration times were used in order to detect low light levels; the toluene detection limit was ca. 0.5 μM and 0.1 μM for a 2 min or a 60 min integration time, respectively.

Naphthalene

The pioneers in the field of luminous microbial biosensors were Sayler and coworkers [45–49] who based their constructs on the *nahG* (salicylate hydroxylase) promoter from the naphthalene degradation pathway of *P. fluorescens*, fused to *V. fischeri's luxCDABE*. King et al. [45] transformed *P. fluorescens* with plasmid pUTK21, which harbored *nahG::lux*, and showed that light output was dependent on naphthalene exposure and degradation rate. The demonstrated application in wastewater and groundwater monitoring suggested the potential future usefullness of the concept. Using the same plasmid, Heitzer et al. [47] developed an optical whole-cell bioluminescence bioassay for specific on-line monitoring of naphthalene and its degradation product salicylate. The reporter bacterium was immobilized in calcium alignate, and the response times were between 8 and 24 min for 120 and 12 μM naphthalene, respectively. The responses to toluene, glucose and to the complex organic growth medium were insignificant.

Matrubutham et al. [48] further experimented with the construct prepared by King et al. [45], immobilized in alginate/$SrCl_2$. Higher luminescence was reached with a complex inducer solution (salicylate plus auxiliary energy supplements) than in the presence of salicylate as the sole carbon source. The lifetime of this sensor was reported to be long (35 days). In general, though responsive to both compounds, *P. fluorescens* HK44 [47] was more sensitive to naphthalene than to salicylate. Apparently, the former was easier to diffuse into the cells as well as serving as a better carbon source [49].

4-Chlorobenzoic acid

A very specific system for 4-chlorobenzoic acid was constructed by Rozen et al. [50]. A fusion of the promoter of the *fcbA* gene of the dehalogenase operon from *Arthrobacter SU* to the *V. fischeri luxCDABE* in *E. coli* resulted in a remarkably specific but insensitive assay.

Aliphatics

The only reported microbial sensor for aliphatics was constructed by Sticher et al. [51], who described the detection of middle-chain alkanes. *E. coli* DH5α was used, with a multi-copy plasmid containing a fusion between the promoter of *alkB* and *luxAB* of *V. fischeri*. The regulatory gene *alkS* from *Pseudomonas oleovorans* was introduced on a separate plasmid. When contaminated groundwater samples were tested, an underestimation of the octane concentration was observed due to unknown inhibitory effects. Induction with n-alkanes from pentane to decane as well as with 3-methylheptane was signifcant, with some response also to long-chain alkanes and dicyclopropylketone. The sensor exhibited a strong dependence on substrate concentration and detection limits were 24–100 nM.

Hydrocarbons

A general system with a broad specificity for hydrocarbons was reported by Selifonova and Eaton [52]. The *ipb* promoter and its regulatory gene, involved in isopropylbenzene catabolism in *Pseudomonas putida RE204*, were cloned in front of the *lux* operon of *Vibrio fischeri*. An *E. coli* strain containing this construct detected a variety of organic molecules including monoalkylbenzenes, substituted benzenes, toluenes, alkanes, cycloalkanes, chlorinated solvents, naphthalenes, gasoline, and cresoate.

Biphenyls

A bioluminescent strain of *Ralstonia eutropha* was constructed for the detection of biphenyls [53]. A 2.8 kb

DNA fragment containing the *orf0-bphA1* section of the biphenyl degradation pathway was cloned upstream of the *V. fischeri luxCDABE* genes. The use of a non-ionic surfactant increased the solubility of the chlorinated biphenyls. Bioluminescence was repressed by biphenyl degradation products such as benzoic acid, but not by its 2-, 3- and 4- chlorinated derivatives. For all tested substances the detection limit was < 6 μM, and the required incubation period varied from 1 to 2 h.

Sensitivities and detection limits

As outlined in the introduction, the principal advantages inherent in biological detection systems lie in their ability to indicate bioavailability, report effects on living systems, and measure global parameters (toxicity, etc.) in a way that no physical or chemical determination is able to attain. The sensitivity and detection limits of the different methodologies, however, are not unimportant, and no bioassay will be environmentally relevant unless it is functional in the correct concentration range. This range is dictated by several factors, including actual contaminant concentrations, environmental regulations, and the limits set by available detection technologies. The detection threshold limits for the various bacterial constructs decribed in this review are listed in Table 2, and can be compared to the data in Table 3, which lists standard environmental techniques as desribed in the technical literature [36]. From such a comparison it is clear that a very broad range of sensitivities is exhibited by the recombinant bacterial sensors, from cases in which the bioassay is far less sensitive than the chemical technique (e.g. in the determination of Co and Cd by atomic absorption spectroscopy) [14, 17, 54], to others in which it equals or even exceeds it [19, 31, 44]. The practicablity of using microbial biosensors for environmental monitoring of specific contaminants thus has to be judged for each case in question. The inexpensive and easy handling of bioassays make their further development attractive. For standard techniques such as gas chromatography which exhibit high sensitivity, drawbacks such as sample extraction, column clean-up, or chemical derivatization before measuring need to be taken into account.

Determination of global parameters

General and oxidative stress (heat shock, katG, uspA)

While most of the bioreporters described above were designed to detect specific chemicals, another group of constructs employs promoters which belong to global regulatory circuits. Since the activities controlled by many of these circuits can be classified as “stress responses”, such strains are expected to respond to a much broader range of environmental changes, mostly ones that are deleterious to the cells. Some of these constructs, therefore, were pro-

Table 3 Standard test methods and detection ranges

Analyte	Test method	Detection range	Reference
Inorganic compounds			
Aluminium	Flame atomic absorption	18–180 μM	[36]
Arsenic	Atomic absorption hydride generation	13 nM–26 μM	[36]
	Atomic absorption graphite furnace	66 nM–1.3 μM	
Cadmium	Atomic absorption direct	445 nM–0.2 μM	[36]
	Chelation-extraction	44.5 nM–2 μM	
	Differential pulse anodic stripping voltammetry	10 nM–1 μM	
	Atomic absorption graphite furnace	18–90 nM	
Chromium	Photometric: Diphenylcarbohydrazide	0.2–10 nM	[36]
	Atomic absorption, direct	2–200 μM	
	Atomic absorption, graphite furnace	100 nM–2 μM	
Copper	Atomic absorption, direct	80 nM–80 μM	[36]
	Atomic absorption, chelation-extraction	800 μM–8 μM	
	Atomic absorption, graphite furnace	80 nM–2 μM	
Iron	Atomic absorption direct	2–90 μM	[36]
	Atomic absorption, graphite furnace	90 nM–1.8 μM	
	Photometric	1–18 μM	
Mercury	Atomic absorption	2.5–50 nM	[36]
Nitrate	Cadmium reduction	3.5–70 μM	[36]
Zinc	Atomic absorption, chelating	76–30 μM	[36]
	Atomic absorption, extraction	0.3–3 μM	
Organic compounds			
Alkane	GC/MS	5–50 nM	[51]
Naphthalene	Fluorescence spectroscopy	0.2 μM	[36]
PCB	Gas chromatography	< 1 μg/L	[36]
Salicylate	Fluorescence spectroscopy	0.4 μM	[36]
Toluene	Fluorescence spectroscopy	0.2/0.1 μM	[36]
Xylene	Fluorescence spectroscopy	0.18–0.13 μM	[36]

posed as tools for ecotoxicological or ecogenotoxicological testing.

E. coli strains containing the promoters of the heat shock genes *grpE, dnaK*, or *lon* fused to *V. fischeri's luxCDABE* responded by enhanced bioluminescence to a variety of chemicals, including metal ions, solvents, pesticides and other organic molecules [55–57]. Fusion of the "universal stress protein" *uspA* gene promoter to the same reporter genes similarly yielded a general stress response, with a similar rank-order of inducers but lower levels of induction [58]. An interesting phenomenon relating to heat shock promoter fusion was the synergistic mode of induction exhibited by pairs of chemicals, most notably when ethanol was one of the two [55].

When *E. coli* strain DE112, containing an outer membrane mutation *(tolC)* was used, enhanced detection of a hydrophobic molecule, pentachlorophenol, was achieved. Maximal response in the *tolC*$^+$ strain was at 0.14 mM, while in the *tolC* mutant it occurred at 4.5 μM [56].

In fusions of the same *lux* genes to a different set of promoters, a sensitive detection of oxidative stress was obtained. Sensing of peroxides was achieved using the promoter of *katG*, one of *E. coli's* catalases, under the control of *oxyR* [59, 60, 62]. For detecion of superoxides, a promoter of the *micF* gene was used [59–61]. This gene is part of the superoxide-activated *soxRS* regulon. The *katG* fusion was shown to be activated by H_2O_2, organic peroxides, redox-cycling agents (methyl viologen and menadione), a hydrogen peroxide-producing enzyme system (xanthine and xanthine oxidase) and cigarette smoke. A synergistic response was observed when cells were exposed to both ethanol and H_2O_2. Luminesence of the *micF* fusion was strongly induced by redox cycling agents such as methyl viologen (paraquat), which generate superoxide radicals. Both sensors were proposed as reporters for intracellular oxidative stress, as a convenient assay system for antioxidant activities, or for monitoring potential environmental oxidative hazards [59–62].

DNA damage and genotoxicity

Another potential use for recombinant bacterial sensors may be as rapid indicators of genotoxicity; this term refers to any potential hazard to DNA integrity, mutagenic as well as non-mutagenic.

RecA, recN, uvrA, alkA

Vollmer et al. [63] desribed a sensor system in which DNA damage-inducible promoters *recA, uvrA, alkA* from *E. coli* were fused to *luxABCDE* of *Vibrio fischeri*. The regulation of the first two genes, which are a part of the bacterial SOS DNA repair system, is *lexA* dependent. The *recA* fusion exhibited the most prominent and sensitive response to mitomycin C, H_2O_2, N-methyl-N′-nitro-N-nitrosoguanidine, ethidium bromide and UV irradiation. A comparison of the multicopy *luxCDABE* and *lacZ* reporter plasmids indicated a greater sensitivity of the former. Further modifications to the same system [64] included integration of the *recA′::lux* fusion into the *E. coli* chromosome, a change of the reporter system to *Photorhabdus luminescens lux*, and the use of either *Salmonella typhimurium* or a *tolC E.coli* mutant as alternative hosts. Application of the *P. luminescens* reporter, which allowed a working temperature of 37 °C, resulted in a more rapid response, as did the use of *S. typhimurium* as a host. The *tolC* mutation increased the sensitivity of induction by mitomycin C but not by hydrogen peroxide. The chromosomal integartion led to a more sensitive response, mostly due to the much lower luminesence background in this single-copy fusion.

A different SOS based approach was that employed in the rec-lac test [65], based on *E. coli* tester strains carrying *recA::lacZ, uvrA::lacZ* or *lexA::lacZ* fusions. A dose-dependent response to all oxidative mutagens tested was observed with the *uvrA*, but not with the *recA* version.

Also based on monitoring SOS activation is the VITOTOX® test, described by van der Lelie et al. [66–68]. The *E. coli recN* promoter was fused to the *lux* operon of *V. fischeri* and introduced into *S. typhimurium*. The *recN* promoter region, which contains two LexA binding sites, is tightly regulated by the LexA repressor. Among the variants tested, the two that performed the best were either with a deleted LexA2 site or with a promoter up mutation. The VITOTOX® test is now commercially available.

The *luxCDABE* genes of *V. fischeri* were also fused to the *recA* promoter of *Pseudomonas aeruginosa* [69]. Light production in response to UV exposure was monitored in a *P. aeruginosa* host, as part of a study of UV effects on natural bacterial populations.

RAD54

A different genotoxicity bioassay was constructed in yeast by Billinton et al. [70] and Walmsley et al. [71] who used GFP as reporter protein and the promoter of *RAD54* as its genotoxin-inducible activator. An induction period of 16 h was necessary, but reagent-free measurement of GFP fluorescence was possible in cell extracts as well as in intact cells [70, 71].

SfiA

Another assay for the detection of DNA-damaging agents is the commercially available SOS Chromotest. It is based on the induction of the SOS gene *sfiA*, monitored by means of a *lacZ* fusion [72, 73]. In view of its simplicity and its rapid response, the SOS Chromotest test was proposed as a complementary or alternative test to the *Salmonella* reverse-mutation assay (the "Ames test") [73]. The assay was shown to detect genotoxines inactive in the Ames test and to allow the identification of false positives.

ColD

Ptitsyn et al. [74] also developed a sensor for genotoxins. The plasmid carries the promoterless *luxCDABFE* genes downstream of the strong SOS *ColD* promoter. The specificity of this luminescent SOS response was demonstrated for different mutagens at a sensitivity ranging from the nanomolar to the micromolar level, depending upon the chemical and the *E. coli* host strain.

Umu

A different SOS induction-based system which is gaining acceptance for the detection of genotoxicants is based on a *lacZ* fusion to the *umu* promoter (the "*umu* test") [75]. The *umu* gene is induced by DNA-damaging agents and is regulated genetically by *recA* and *lexA*. The test organism *S. typhimurium* TA1535 has excision repair deficiency *(uvrB)*, increased membrane permeability *(rfa)*, and a natural deletion of the *lac* operon. For many tested mutagens enzyme synthesis and activity reached a plateau within 2 h, and the sensitivity was comparable to that of the Ames test. The test allows the assaying of chemicals which may be too toxic for the standard Ames test.

Toxicity

The most widely accepted microbial toxicity test system is marketed as Microtox® [76, 77] and is based upon the wild-type luminescent bacterium *V. fischeri*. Sample toxicity is assessed from the decrease in luminescence following a short exposure (normally 15 min) to several concentrations of the sample. A similar approach to ecotoxicity testing using a genetically engineered bacterium was reported by Lampinen et al. [78], who cloned the *lux* gene from *Vibrio fischeri* under the control of the *lac* promoter. Reported performance for the chemicals tested appeared similar to that of the Microtox assay.

A different approach for toxicity assessment was proposed by Belkin and coworkers, who employed a set of *E. coli* strains harboring different plasmids, each carrying a different stress promoter::*lux* fusion [79, 80]. The panel members included oxidative stress sensors (*micF* for superoxides and *katG* for peroxides), a DNA damage sensor *(recA)*, and two "general stress" strains with the *grpE* and *fabA* promoters. The latter is the only panel member not mentioned earlier in this review; the *fabA* gene, coding for β-hydroxydecanoyl-ACP dehydrase, a key enzyme in the synthesis of unsaturated fatty acids, is under the control of the *E. coli* regulator FadR. Like the heat shock promoters, it responded to a large variety of chemicals and proved to be a general toxicity indicator. The responses of the panel members allowed one to distinguish between oxidative, DNA damaging and general toxic effects. Enhanced sensitivities to different groups of compounds were observed, as well as to industrial wastewater samples. In some cases the response spectra were broader, and phenolic compounds, for instance, induced most if not all of the strains.

The general approach for using promoter::*lux* fusions for the detection of diverse environmental stress factors, including general toxicity and genotoxicity, has been established in several patents [67, 81, 82].

Conclusion and future perspectives

Physical and chemical methodologies for analyzing pollutants in environmental samples – though often costly and complex – are highly accurate and sensitive, and are indispensible for the maintenance of environmental standards of all types. These approaches fail, however, to provide information on the bioavailability of the analyzed compounds, or as to their effects on biological systems.

Not surprisingly, therefore, the last decade has seen an impressive development of diverse bioassays designed to fill this gap. As demonstrated by the data in this review, recombinant bacteria play a major role in this field, and the potential inherent in their use is very broad. Nevertheless, it is also apparent that at present the applicability of most of these systems is still limited. There is a great variability in response times, detection thresholds, sensitivity, signal relaxation lengths and stability. There is also a pronounced lack of information concerning the functionality of the microbial biosensors in "real" samples to which they may be subjected. Further study is obviously needed in order to improve performance and stability; part of this effort has to be dedicated to attempts at a better understanding of the molecular controls of both the induction and expression of the desired activities. This basic information is essential for improved design and construction of the bioassay strains.

It is also clear from the data presented here that such improvements and basic studies are continuously being sought, and that the field is indeed progressing towards better genetic constructs. It is therefore to be expected that in the years to come some of these will be recognized as increasingly important tools for environmental biomonitoring. The approach is already commercially established in the detection of certain contaminant groups, such as genotoxicants.

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