Microbial whole-cell sensing systems of environmental pollutants
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The past decade has witnessed the development of a novel class of tools for environmental monitoring: genetically engineered microorganisms ‘tailored’ to respond in a dose-dependent manner to changes in environmental conditions. Recent advances in the field include the expansion of available reporter functions with multicolored fluorescent proteins, a broadening of the detected chemical effects such as the availability of nutrients and enhancement of the spectrum of reporter microorganisms to include cyanobacteria, yeast and fungi. Most importantly, the stage has been set for the incorporation of such cells into various whole-cell array formats on silicon chips, optic fibres and other configurations. The future of such multiplex detection and analysis systems seems bright.

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Introduction
There are two general approaches for monitoring chemicals in the environment. The traditional approach is based on chemical or physical analysis and allows highly accurate and sensitive determination of the exact composition of any sample. It is essential for regulatory purposes and is necessary for understanding the causes of pollution and the means for its potential remediation. However, a complete array of analytical instrumentation necessary for such exhaustive analysis is complex, costly and requires specialized laboratories. In addition, such methodologies fail to provide data on the bioavailability of pollutants, their effects on living systems and their synergistic or antagonistic behavior in mixtures. As a partial response to these needs, a complementary approach is based on the use of living systems in a variety of environmentally oriented bioassays.

Numerous biological systems have been used for such purposes, ranging from live-organism assays such as fish toxicity tests to others based on sub-cellular components or enzymes. All of these assay the effect of the target chemical(s) rather than identify the chemical itself. Unicellular microorganisms, in particular, bacteria, are advantageous for such purposes. Their large population size, rapid growth rate, low cost and easy maintenance make them a lucrative option for pollution monitoring. An additional attractive characteristic of bacteria is that they can be ‘tailored’ to respond by a detectable signal to specified changes in their environmental conditions.

Several recent reviews [1–5,6] have addressed different aspects of the use of such genetically engineered microorganisms as environmental bioreporters. In this review, I highlight the most recent advances in this rapidly developing discipline.

Biosensors, whole-cell biosensors and environmental monitoring
A general definition for the term ‘biosensor’ is ‘the coupling of a biological material with a microelectronic system or device to enable rapid, accurate, low-level detection of various substances in body fluids, water and air’ [7]. The obvious advantage of using biological material is that biological molecules are extremely highly specific. Successful biosensors have been based on the specific interactions between enzymes and their substrates, the recognition between antibodies and antigens, accessibility of specific target molecules to their receptors, or the high affinity of nucleic acid strands to their complementary sequences. In all of these cases, the focus is on the specificity endowed by the unique biorecognition of two molecules.

In a completely different analytical approach, a live, intact cell is used as the biological entity. Although much of the specificity described above might be lost, it is more than compensated for by the fact that in this manner we are able to detect, by very simple means, a very complex series of reactions that can exist only in an intact, functioning cell. Global parameters such as bioavailability, toxicity and genotoxicity (damage to genetic material) cannot be probed with molecular recognition or chemical analysis; they can be only assayed using whole cells. In view of the direct relevance of bioavailability and toxicity to the presence of pollutants, many of the efforts at the
development of whole-cell biosensors were directed towards environmental applications.

Environmental toxicity bioassays: ‘lights off’ and ‘lights on’

As implied above, the obvious strength of whole-cell biosensing is not in the specificity of the observed responses but rather in their generality. This is most apparent in toxicity bioassays, designed to assess the sum negative impact of the sample on living systems. In such assays, the question asked is not ‘what toxicants does the sample contain?’ but rather ‘how toxic is the sample?’

In recent years, genetically engineered microorganisms have been used in two different ways in the development of toxicity bioassays. I shall address them as ‘lights off’ and ‘lights on’ assays. The differences between the two concepts are schematically represented in Figure 1.

The ‘lights off’ assay is an extension of the widely accepted microbial toxicity bioassay, which is based on the measurement of a decrease in light emission as a function of sample concentration by the wild-type luminous bacterium *Vibrio fischeri* following a short-term exposure to the sample [8]. A drawback often mentioned with regard to this assay is the marine origin of the test organism. Consequently, in the search for more ‘environmentally relevant’ systems, other microorganisms have been modified to constitutively luminesce and thus serve as more realistic indicators of environmental toxicity. Examples include *Escherichia coli* HB101 (harboring luxCDABE of *V. fischeri*) immobilized in polyvinyl alcohol [9], *Pseudomonas fluorescens* transformed with the same plasmid [10], or the cyanobacterium *Synechocystis* PCC6803 marked with *luc* from the firefly *Photinus pyralis* [11]. In the latter case, the luciferase substrate firefly luciferin had to be added externally. Using a different approach, Ulitzur et al. [12] reported the use of a ‘highly sensitive variant’ of the marine bioluminescent *Photobacterium leiognathi*. Weitz et al. [13] demonstrated the use of two naturally bioluminescent fungi, *Armillaria mellea* and *Nycena citricolour*, for the same purpose.

A somewhat more sophisticated approach to pollution-effect bioassays is based on the molecular fusion of a reporter system to selected promoters of different stress-response regulons. Assuming that no single reporter strain will be able to cover all potential cellular stress factors, it has been proposed that a panel of such strains be used [14]. Similar panels have recently been shown to sensitively respond to important environmental pollutant classes such as dioxins [15] and endocrine disruptors [16]. In these and many similar studies, the reporter system of choice was microbial bioluminescence (luxCDABE), hence the reference to the ‘lights on’ assays (Figure 1).

In a similar manner, bacterial strains have also been developed for assaying genotoxicity, rather than ‘regular’ toxicity. In these cases, the promoters serving as the sensing elements were selected from DNA repair operons such as the SOS system, and the reporters were either bacterial *lux* or β-gal [4]. Two recent reports [17,18] propose the use of a green fluorescent protein (GFP) gene from the jellyfish *Aequorea victoria*, as an alternative reporter system for the same purpose. A more developed

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**Figure 1**

Bacterial toxicity assay principles. In ‘lights off’ assays (a), sample toxicity is estimated from the degree of inhibition of a ‘normally on’ activity. Such inhibition can occur along any stage of the reaction’s development or in any site affecting cellular wellbeing. In ‘lights on’ assays (b), a quantifiable molecular reporter is fused to specific gene promoters, known to be activated by the target chemical(s).

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www.current-opinion.com  Current Opinion in Microbiology 2003, 6:206-212
yeast-based *Saccharomyces cerevisiae* GFP system for genotoxicity assessment is being continuously improved by Walmsley and co-workers [19].

**Detection of specific classes of pollutants**

Since the pioneering work of Sayler and co-workers in the construction of a *lux* fusion for the specific detection of naphthalene and salicylate [20], there has been a steady stream of similar constructs responsive to distinct organic and inorganic pollutants or classes of pollutants (reviewed recently in [1–4,5*–6]). Bioluminescence has served as the reporter in most cases, with a few examples of β-galactosidase activity and, more recently, GFP accumulation. Recent additions to these bioreporter families include a bioluminescent phenol-sensing *Acinetobacter* [21], a GFP-based toluene-responsive *Pseudomonas* [22] and a β-gal reporter of 3-chlorocatechol [23].

An important point often ignored in this context is that almost without exception, standard analytical chemical techniques are likely to provide more sensitive, accurate detection and quantification of specific pollutants than the microbial reporters [4]. The main advantage offered by the microbial reporters is that they can sense only the bioavailable fraction of the detected chemical, thus allowing its differentiation from the non-available fraction. Such information might be highly valuable for risk assessment and for the selection of suitable remediation options (see also Update).

**Nutrient bioavailability**

An interesting offshoot of environmental promoter–reporter fusions was the successful attempts, carried out independently in several laboratories, at the construction of cyanobacterial bioreporters of nutrient bioavailability. Although wastewater nitrogen and phosphorus are not the first on the ‘most wanted’ pollutant list, they are nevertheless a primary cause of eutrophication in aquatic environments and the direct trigger for the development of algal and cyanobacterial blooms. It was therefore of interest to determine how much of the limiting nutrient is actually seen by phytoplankton cells, as opposed to the total concentration determined by chemical means.

Using similar strategies, Gillor *et al.* [24] and Mbeunkui *et al.* [25] reported on a *Synechococcus* sp. with a glnA:*lux* fusion and a *Synechocystis* sp. with a nblA:*lux* fusion, respectively, both of which are sensitive reporters of available nitrogen. The *Synechococcus* glnA reporter was responsive to ammonia, nitrate, nitrite and organic nitrogen, whereas the *Synechocystis* nblA reporter was characterized mostly for nitrate detection. Gillor *et al.* also reported a highly sensitive biosensor for bioavailability sensor based on a phoA:*lux* fusion in *Synechococcus* sp. [26*]. Using this strain, under phosphorus limitation, ‘bioavailable phosphorous’ constituted 1% or less of the chemically determined element in a freshwater lake (Gillor *et al.*, unpublished). Similarly, to assess the bioavailability of iron, another highly significant element for primary producers, Durham *et al.* [27] fused *Vibrio harveyi* luxAB genes to the isiAB promoter of *Synechococcus* PCC 7942 and reported a sensitive dose-dependent response (see also Update).

**Bioluminescence or fluorescence?**

Of the genes used over the years as the reporting elements in environmental microbial sensor systems, bacterial bioluminescence genes have played a prominent role. During the past few years, as increasingly versatile fluorescent protein genes have become available for general use [28*,29**], their popularity as reporters has also increased. Recently, at least two studies [17,30] compared bioluminescent with fluorescent reporting and came up with very similar conclusions. Bioluminescence allows a much faster and more sensitive detection of the target analyte than fluorescence. This is not surprising as bioluminescence is a measure of enzymatic activity, whereas fluorescence quantifies the presence of the protein. The advantage of using GFP and other fluorescent proteins is in their very high stability. When real-time monitoring of the developing signal is impractical, fluorescence might prove to be much more reliable; if sufficient time is allowed for signal accumulation, even the sensitivity will be significantly improved [17]. It is not unlikely that future improvements in available fluorescent protein reporters will close this gap.

**Dual labeling**

Although it seems an intriguing objective, there have been very few reports on the inclusion of two distinct reporters in a single organism. In an early report, Wood and Gruber [31] introduced two beetle luciferases, different in their light-emission spectra, into *E. coli*. A similar study was recently reported in which two fluorescent proteins (GFPuv and YFP) were introduced into the same bacterial host species [32]. In both cases, one of the reporters served as the responder to the analyte and the other as an internal control.

We have recently constructed a different double-labeled *E. coli* construct, containing two inducible promoters fused to different fluorescent protein genes: recA':egfp and gfpE::dsRedExpress. The recA promoter is a part of the bacterial SOS system and its activation is considered an indication of DNA damage [33]. GrpE is a heat-shock protein, induced by a broad spectrum of chemicals, and is an excellent indicator of toxic cellular stress [34]. Their combination, therefore, potentially allows an assay of both genotoxicity and ‘regular’ toxicity by the same organism. Indeed, when separately challenged with model chemicals, nalidixic acid (an SOS activator) and ethanol (a heat-shock inducer), the strain responded by an enhanced expression of the expected protein (Figure 2). A detailed description of this construct will be published elsewhere.
Immobilization and integration into biosensors

Among microbiologists, the term ‘biosensor’ or ‘microbial biosensor’ is often used to describe only the responsive microbial strain. In biosensor literature, however, it is claimed that to be considered a true biosensor the biological entity needs to be integrated into the appropriate hardware, as indicated in the definition at the beginning of this review. Although the isolated bacterial strain might serve as an excellent reagent in the laboratory, to be taken outside its boundaries it needs to be incorporated into a device that will allow storage and maintenance of the live cells, access to the sample and signal capture. Several recent reports offer some potential incorporation solutions including alginate attachment onto optic fibre tips [35*], agar immobilization at the bottom of microtiter plate wells [25] and encapsulation in sol-gel matrices [36]. In the latter report, encapsulated luminescent bacteria maintained full activity at 4°C for over three months. Figure 3 displays a confocal image of two sol-gel encapsulated E. coli strains, each containing a different fluorescent protein gene (GFPuv, green fluorescence, or DesRed2, red fluorescence) fused to the recA promoter. The strains were co-immobilized in a 160 μm thick sol-gel film as previously described [36], and induced in the sol-gel by mitomycin C (1.2 μM). Images were recorded 17 hours after induction. The micrograph shows that both strains were alive and inducible inside the sol-gel matrix.

Responses to (a) a ‘regular’ toxicant (ethanol, 6%) and (b) a genotoxicant (nalidixic acid, 6 mg/l) of a double-labeled E. coli reporter. The plasmid in this construct harbors both a recA::egfp (green fluorescence; excitation 488 nm; emission 535 nm) and a grpE::DsRed Express (red fluorescence; excitation 558 nm; emission 583 nm). Fluorescent protein genes were obtained from BD Biosciences Clontech.
A different approach was reported by Biran and Walt [37**]. Individual fluorescent *E. coli* and *S. cerevisiae* cells were randomly embedded into a high-density microwell array, etched at the distal edge of an optical imaging fiber. The location and fluorescence of each individual cell was monitored using an optical decoding system, based on the specific ‘signature’ of each cell type. A chip-based system was developed by Simpson and co-workers, who used a complementary metal-oxide semiconductor (CMOS) imager for very low-level detection of the bioluminescent signal of a *Pseudomonas fluorescens* strain induced by naphthalene or salicylate [38]. Their device, termed ‘bioluminescent bioreporter integrated circuit’ (BIBIC), is probably the first integrated whole-cell biochip.

A different tack towards the production of a whole-cell biochip is pursued in our laboratory, in which fluorescent *E. coli* sensor cells are maintained in millimeter-size cavities on a silicon chip. The fluorescence induced following an introduction of the sample into the cavity is recorded by a CMOS imager and quantified. Figure 4 displays an on-chip image of a *recA*::*egfp* harboring strain, induced by nalidixic acid.

**Whole-cell arrays**

In recent years, there have been dramatic advances in a new analytical format — the microarray, a tool that has revolutionized our ability to characterize and quantify biologically relevant molecules. The principle in all cases is the same: a large family of well-defined reactive molecules is fixed onto a mapped solid surface grid and exposed to a multi-component analyte mixture. Sites on the chip in which a recognition event has occurred (e.g. by a complementary nucleic acid sequence) are identified by one of several possible detection techniques such as fluorescence; the characteristics of the sample can then be discerned from the nature of the bioreceptor molecules occupying these sites. Using this principle, an increasingly large number of applications are being developed in medicine, biology, toxicology, drug screening and more.

Most of the arrays described are based on DNA (‘DNA chips’), some on proteins or antibodies, and a fewer number on other biological entities such as enzymes. Very recently, the idea of whole-cell arrays has been advanced by Van Dyk and co-workers [39**] who described the LuxArray: a collection of 689 non-redundant functional promoter fusions to *Photorhabdus luminescensluxCDABE* in live *E. coli* strains, representing close to 30% of the predicted transcriptional units in this bacterium. High-density printing of the reporter strains to membranes on agar plates was used for simultaneous assays of gene expression with impressive results. Although presently aimed more towards gene expression studies, there is no doubt that this approach can be used successfully for environmental monitoring, efficiently combining effect-testing with analyte identification.

**Conclusions**

A decade of genetically engineering microorganisms for the detection of either toxic effects or of specific classes of chemicals has set the ground for a new and exciting era. The potential incorporation of such cells into numerous array formats on biochips, optic fibres, or other suitable surfaces, will allow a mode of bioanalysis previously considered impossible. A positive response of a live-cell array will indicate the existence of the probed effect, the response pattern will indicate the identity of the chemicals in the sample and the intensity of the response will quantify their concentrations. In other configurations, such arrays can serve for high-throughput screening of chemicals and drugs, simultaneously monitoring both for desired and negative effects.

**Update**

Whole-cell microbial sensors have recently been used in several studies to address the relatively untouched topic of bioavailability of chemicals in soils. Standing *et al.* [40] presented a trio of bioluminescent *Pseudomonas fluorescens* strains for assaying carbon, nitrogen and phosphorus in soil. One of these strains was also used to assay carbon substrate exudation from *Hordeum vulgare* roots [41]. Burmølle *et al.* [42] used a somewhat surprising yet rewarding approach to assay the presence of *N*-acyl homoserine lactone in soils: they fused a quorum-sensitive lux promoter of *V. fischeri* to a GFP reporter in *E. coli*. A different twist to the accepted rationale of biosensor engineering was presented by Casavant *et al.* [43], who used site-specific recombination-based GFP microbial reporters (*Pseudomonas fluorescens* and *Enterobacter cloacae*) for detecting toluene and related compounds in root environments. Finally, Turpeinen *et al.* [44], studying arsenic bioavailability in soils with an arsenic-specific bioluminescent
E. coli strain, addressed issues often ignored by others such as time-dependent metal sequestration.

Acknowledgements

Research in the Belkin laboratory was supported by the Defense Advanced Research Projects Agency (DARPA) of the US Department of Defense (Grant N00173-01-1-G009) and by the State of Niedersachsen (Germany) grant number 16.11.1998–25 A.S.76 251-99-298 (ZN549). The confocal micrograph (Figure 3) was provided by O Lev and R Premkumar (Hebrew University of Jerusalem, Israel) and the CMOS images (Figure 4) by R Rosen (Hebrew University) and Y Shachm (Tel Aviv University, Israel). Construction of the double fluorescent E. coli reporter was carried out by N Hever in the framework of her MSc. thesis.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

• of special interest
•• of outstanding interest


Together with Gillor et al. (2003) [24], Mbeunkui et al. (2002) [25] and Durham et al. [27], this article describes a novel approach to assaying the levels of essential nutrients as ‘seen’ by phytoplankton cells. These levels may differ significantly from the much higher concentrations of the same nutrients determined by standard analytical means.


An updated and compact review that highlights some of the potential applications of fluorescent proteins in dynamic studies of microbial phenomena.


A review of ‘state of the art’ fluorescent protein usage in real-time imaging of live cells, which also outlines some of the paths along which this revolution will proceed.


The importance of this description of the encapsulation of live cells in sol-gel matrices is in the unprecedented shelf life of the immobilized cells: over 3 months with no loss of activity.


In what might be a breakthrough in whole-cell sensor technology, the authors describe the deposition of individual cells in micrometer-size cavities at the end of an optical imaging fibre and monitor their individual fluorescent responses.


One of a series of articles by Simpson, Sayler and co-workers that may pave the way for the practical whole-cell biochips of the future.


Highlighting one of the future directions by which whole-cell arrays will surely advance, this article describes a collection of bacterial strains, harboring lux fusions to different promoters. The intensity and pattern of the responses provide a wealth of data on sample composition and its effects.


