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Review

Recent advances in biosensor techniques for environmental monitoring

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Abstract

Biosensors for environmental applications continue to show advances and improvements in areas such as sensitivity, selectivity and simplicity. In addition to detecting and measuring specific compounds or compound classes such as pesticides, hazardous industrial chemicals, toxic metals, and pathogenic bacteria, biosensors and bioanalytical assays have been designed to measure biological effects such as cytotoxicity, genotoxicity, biological oxygen demand, pathogenic bacteria, and endocrine disruption effects. This article is intended to discuss recent advances in the area of biosensors for environmental applications.
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Keywords: Biosensors; Bioassays; Environmental monitoring

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1. Introduction

Monitoring of contaminants in the air, water and soil is an instrumental component in understanding and managing risks to human health and the environment. Given this requirement as well as the time and cost involved in traditional analytical chemical analysis of environmental samples, there is an expanding need for simple, rapid, cost-effective and field portable screening methods. Biosensors and bioanalytical methods appear well suited to complement standard analytical methods for a number of environmental monitoring applications.

The definition for a biosensor is generally accepted in the literature as a self contained integrated device consisting of a biological recognition element (enzyme, antibody, receptor or microorganism) which is interfaced to a chemical sensor (i.e., analytical device) that together reversibly respond in a concentration-dependent manner to a chemical species [1] (Fig. 1).

Although the generally accepted definition of a biosensor requires a direct interface between the biological recognition element and signal transducer, a wide range of bioassay formats including genetically engineered microorganisms that respond in observable ways to target analytes are frequently referred to in the literature as bioreporters or biosensors. Because many of these bioassays show the potential for development as biosensors, these tech-

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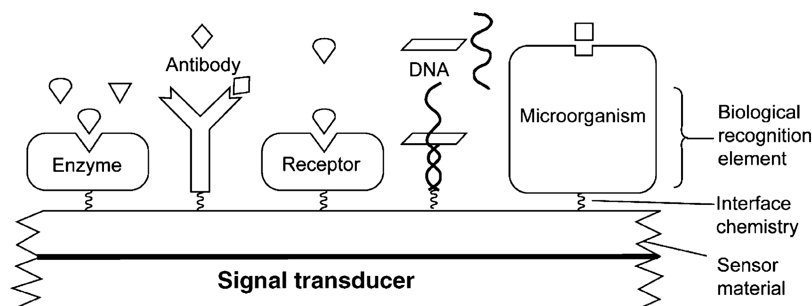

Fig. 1. Schematic representation of biosensors.

niques will be included for the purpose of the present discussion.

The use of biosensors for environmental applications has been reviewed in considerable detail [2]. In addition, biosensor technology has been recently reviewed from the perspectives of agricultural monitoring [3], ground water screening [4], ocean monitoring [5] and global environmental monitoring [1]. The intention of this article is to discuss recent advances and trends in the use of biosensors and related bioanalytical assays for environmental monitoring applications. The trends and areas of advancement for various biorecognition elements are summarized in Table 1.

2. Enzyme-based biosensors

A wide range of biomolecular recognition elements have been used for biosensors for potential environmental applications. These can be organized by structural (e.g., enzyme, antibodies or microorganisms) or functional (e.g., catalytic, affinity or complex cellular functions) characteristics. Enzymes were historically the first molecular recognition elements included in biosensors and continue to be the basis for a significant number of publications reported for biosensors in general as well as biosensors for environmental applications. There are several advantages for enzyme biosensors. These include a stable source of material (primarily through biorenewable sources), the ability to modify the catalytic properties or substrate specificity by means of genetic engineering, and catalytic amplification of the biosensor response by modulation of the enzyme activity with respect to the target analyte. There are also some limitations for enzyme-based biosensors with respect to environmental applications. These include the limited number of substrates for which enzymes have been evolved, the limited interaction between environmental pollutants and specific enzymes, and in the case of inhibitor formats, the lack of specificity in differentiating among compounds of similar classes such as nerve agents as well as organophosphate (OP) and carbamate pesticides.

Recent progress with respect to enzyme biosensors for environmental applications has been reported in several areas. These areas include the following; genetic modification of enzymes to increase assay sensitivity, stability and shelf life; improved electrochemical interfaces and mediators for more efficient operation; and introduction of sampling schemes consistent with potential environmental applications.

Genetically engineered acetylcholinesterase (AChE) variants from *Drosophila melanogaster* have shown inhibition constants for the insecticide methamidophos that were up to three orders of magnitude higher than for commercially available AChE from *Electrophorus electricus* (Eel) [6]. Variants were more or less sensitive to the particular insecticide suggesting that certain variants may be selective for other related compounds. The genetic modification approach to *D. melanogaster* AChE has also been used to construct a microporus-activated carbon electrode biosensor for diclorvos with a reported detection limit of 10^{-17} M which is four orders of magnitude lower than a similar biosensor using AChE from Eel [7]. In another report, a biosen-

Table 1
Areas of advancement for biorecognition elements

Bio recognition element	Area of advancement	Reference
Enzyme	Genetic modification for improvement of sensitivity and stability	[6–8]
	Improvement of sensor interface	[9–17]
	Improvement of operational format and unique environmental applications	[18–26]
Antibody	Multi-analyte detection	[27–31]
	Automation and demonstrated environmental applications	[30,31]
	Reversible binding and simplified or improved format	[32–42]
Cell-based	Novel gene fusions responsive to specific compound classes and assay format improvements	[14–48,54,55]
	Novel gene fusions responsive to toxic or genotoxic stressors	[49–53]
	Native organisms responsive to specific compounds, toxic stressors and biological oxygen demand	[44,56–69]
DNA	DNA damage	[71–78]
	DNA hybridization and detection of pathogens	[79–85]
Receptors	Estrogenic and endocrine disrupting compounds	[18,19,31,86–90]

sor constructed from a genetically modified AChE variant from *D. melanogaster* was inhibited by the insecticide omethoate with a limit of detection (LOD) of 10^{-17} M [8]. This finding was of particular significance in that the AChE from Eel was not inhibited by omethoate, suggesting that it may be possible to select an array of variants that would respond to specific insecticides.

One important step in biosensor development is immobilization of the biological recognition element to the sensor surface. A number of innovative immobilization techniques have been reported using enzymes. Approaches for these techniques include the use of new materials and incorporation of oxidation–reduction (redox) mediators into the immobilization process. Anionic clays such as Zn-Cr-2,2″-azinobis-3-ethylbenzenothiazoline-6-sulfate (ABTS) have been used to immobilize horse radish peroxidase (HRP) onto glassy carbon electrodes used as biosensors to measure cyanide [9]. In this configuration, the electroactive anions act as an electron shuttle between the redox center of HRP and the electrode surface. In another variation of this scheme, polyphenol oxidase (PPO) was immobilized on a glassy carbon electrode using Zn–Al layered double hydroxides (anionic clay) [10]. This biosensor was extremely sensitive to cyanide with a detection limit of 0.1 nM.

Another innovation for immobilization of enzymes to biosensors involves the use of sol–gel. Advantages for this strategy include thermal stability, pH buffering and physical ruggedness typically required for environmental applications. One recent example of this approach includes co-immobilization of urease and FITC-dextran as a fluorescent reporter into sol–gel [11]. Inhibition of enzyme activity was used to measure Cu(II) and Cd(II) both over the concentration range of 20–230 μM. In another example, AChE and powdered carbon were doped into sol–gel to form an electrochemical biosensor [12]. This enzyme electrode was inhibited by oxydementon methyl over the concentration range of 2–200 ppb and could be re-used for over 60 measurements.

Other approaches for immobilization of enzymes to environmental biosensors include combinations of covalent and non-covalent binding. High affinity binding of a glycoprotein enzyme (AChE) to a concanavelin A (Con A)-activated screen printed carbon ink electrode [13] yielded a high sensitivity response for chlorpyriphos and good operational stability. Both covalent and non-covalent immobilization of AChE to a polyethyleneimne-modified screen printed electrode resulted in the maintenance of enzyme activity on a dried electrode up to 1 year [14].

Advances in processes and particularly in the area of electrochemistry have yielded improvements in operational characteristics of biosensors. For example, the sonochemical ablation of a non-conducting polymer-coated electrode produced microelectrode arrays [15,16]. Bioengineered AChE was then immobilized in polyanaline at the microelectrode surface forming mushroom-like structures. This biosensor was highly sensitive to the pesticides dichlorvos, parathion and azinphos which were detected at concentrations as low as 10^{-17}, 10^{-16}, and 10^{-16} M, respectively [15].

Nanomaterials have also been used to improve the operational characteristics of enzyme-based biosensors. This improvement results from both increased surface area and increased catalytic activity. For example, the measurement of hydrogen peroxide produced during the catalysis of acetylcholine by an acetycholinesterase-choline oxidase biosensor was significantly improved by immobilization of carbon nanotubes onto carbon ink screen printed electrodes [17]. This biosensor was sensitive to several OP insecticides.

Another promising development in the area of enzyme-based biosensors is the observation that a number of structurally related and environmentally significant phenols with endocrine disrupting compounds are also substrates for the enzyme tyrosinase. The tyrosinase–carbon paste electrode which has been extensively characterized for phenol and catechol substrates has also been recently used to measure endocrine disrupting compounds such as quercetin, resueratol, genistein and bisphenol A at concentrations in the low μM range [18].

Further advances to tyrosinase–carbon paste electrodes have been demonstrated by trapping the enzyme into a cross-linked redox mediator phenothiazine [19]. This enzyme electrode responded to endocrine disrupting compounds such as 17-α-ethineloestradiol, 1-β-oestradiol, nonylphenol and bisphenol A in the sub μM concentration range.

A number of enzymes have been shown to be inhibited by toxic metals that contaminate the environment. Limitations for the potential application of enzyme biosensors have included limited sensitivity, limited selectivity as well as interference by environmental matrices and non-metal inhibitors. Strategies that have been used to improve sensitivity and selectivity include the use of ratio measurements for multiple enzymes, changes in signal transduction (e.g., use of amperommetry as opposed to potentiometry) and examination of enzymes not typically used for inhibition format biosensors. More specifically, the enzymes that have typically been used include urease and AChE. Signal transduction is often accomplished by following inhibition in the rate of enzyme catalyzed pH change caused by toxic metals such as Hg(II), Cu(II), Cd(II), or Zn(II).

One recently introduced strategy uses the ratio of AChE/urease as measured by FITC-dextran (a fluorescent pH indicator) [20]. The analysis of inhibition ratios was used to measure Cd(II), and Cu(II), and showed detection limits in the nM range for Hg(II).

Urease inhibition by heavy metals has been typically measured using potentiometric transduction resulting from changes in pH. By contrast, the use of the linked enzyme system of urease and glutamic dehydrogenase has facilitated the measurement of urease through the amperommetrically monitored consumption of NADH (cofactor for glutamic dehydrogenase) [21]. This linked enzyme bioassay was compatible with biosensor development and has been used to measure Hg(II), Cu(II), Cd(II), and Zn(II) at concentrations in the ppm–ppb range. The chromate reductase activity of cytochrome C3 from sulfate reducing bacteria was used to construct a Cr(VI) selective biosensor [22]. This enzyme electrode was responsive to Cr(VI) at concentrations between 3.8 and 132 μM and was not affected by the potential interferants As(V), As(III) or Fe(III). An enzyme-inhibition assay using nitrate reductase has been shown to detect Cd(II), Cr(III), Cr(VI), Cu(II), Ni(II), Pb(II), and Zn(II) in the low μM concentration ranges [23]. The interesting feature for this col-

orimetric bioassay is that the enzyme inhibition by the heavy metals was prevented in most cases by using the chelating agent EDTA which allowed differentiation between heavy metal and non-heavy metal inhibitors.

Although DNA is not typically considered to be a biocatalyst, specific sequences with enzymatic activity have been reported [24]. For this biocatalytic assay, a double stranded segment of DNA was selected which acted as a Pb^{2+}-dependent enzyme that cleaved a partially complementary substrate strand at a specific cleavage site. This DNAzyme activity was used to construct a lead ion assay by incorporating fluorescent indicator and quencher dyes in close proximity on the substrate and catalytic strands, respectively [24]. After lead-dependent cleavage of the substrate strand, the shorter complementary sequences dissociated under the assay conditions and the fluorescence indicator and quencher dyes were physically separated resulting in the measured increase in fluorescence signal. This DNAzyme has also been configured into gold nanoparticle absorbance and visible color change assays [25]. For these assay formats, oligonucleotides that were complementary to sections on the substrate strand were attached to the gold nanoparticles. This allowed the uncleaved substrate sequences to form aggregates. Lead-dependent cleavage of the substrate strands caused the nanoparticle aggregates to disperse with consequent color change.

Although enzyme-based biosensors show significant promise for certain environmental monitoring tasks, they also show several inherent limitations. Their main limitation involves their lack of versatility. For example, inhibition based biosensors detect specific groups of compounds (e.g., cholinesterase biosensors detect OP and carbamate insecticides) with different sensitivities for each compound and urease is inhibited by four or five toxic metals again each with their own concentration range. Unfortunately, environmentally polluted media typically contain many compounds of concern from a number of compound classes. Consequently, environmental applications for enzyme-based biosensors will likely be limited to screening of industrial waste water, surface water or sewage treatment streams where the identity of the compounds of interest are known and an indicator compound or summation of indicator compounds can be linked to an industrial process. One example of a potential application for an enzyme-based biosensor involves a flow injection system with a laccase electrode detector. This biosensor is sensitive to μM concentrations of phenol, *p*-chlorophenol, guaiacol and chloroguaiacol which are compounds of environmental concern in wastewater from paper mills using the Kraft process [26].

3. Antibody-based biosensors

Antibody-based biosensors (immunosensors) are inherently more versatile than enzyme-based biosensors in that antibodies have been generated which specifically bind to individual compounds or groups of structurally related compounds with a wide range of affinities. There are, however, several limitations in the use of antibody-based biosensors for environmental monitoring applications. These limitations include the complexity of assay formats and the number of specialized reagents (e.g., antibodies, antigens, tracers, etc.) that must be developed and characterized for each compound and the limited number of compounds typically determined in an individual assay as compared to the multiple compounds that contaminate environmental samples.

Recent advances reported for antibody-based biosensors for environmental applications have primarily been focused toward these limitations. For example, the simultaneous detection of six hazardous bacteria and protein toxins was demonstrated on a planar waveguide array biosensor [27]. The biohazards included ricin, cholera toxin, *F. tularensis, B. adortus, B. anthrasis* and eneterotoxin B from *S. aureus* in the presence of environmental contaminants such as sand, clay, pollen and smoke. The instrumentation was automated and the assay was compatible for development as a field assay.

In another system, using a microchip format, six toxins, ricin, viscumin, staphylococcal enterotoxin B, tetanus toxin, diphtheria toxin and anthrax toxin were detected at ng/mL levels [28]. The antibodies were immobilized on the glass chip using hydrogel-based chemistry and the toxins quantitated using a fluorescence microscope equipped to measure four wavelengths with a CD camera.

In contrast to the spatially separated micospots or microchambers, another approach to multi-analyte determination uses four-color quantum dot-labeled antibody reagents and four capture antibodies in a sandwich assay format detected in microwell plates [29].

Multi-analyte analysis has also been demonstrated using the fully automated optical biosensor termed the River Analyzer (RIANA) [30]. This system is based on a total internal reflectance fluorescence platform and uses a competitive immunoassay format to simultaneously measure atrazine, bisphenol A and estrone. A detection limit below 0.02 μg/mL for each of the compounds was achieved in matrices such as purified water, ground water and tap water. This method also showed a high degree of reproducibility, precision and robustness.

An automated water analyzer computer supported system (AWACSS) has been recently reported that is capable of measuring 20 analytes in surface, ground, drinking, and waste water [31]. This in-line immunosensor system is remote controlled and fully automated. Analytes that can be measured include a wide range of commonly used pesticides, endocrine disruptors and industrial pollutants with detection limits for many of these compounds being reported in the low ng/L range.

Techniques that are typically used to immobilize analyte specific immunochemicals to specific locations in a sensor array involve physical means such as deposition using micropipettes [28], inkjets [30], or microfluidics [27]. In an alternate approach to spatial positioning of immunochemicals, an electric field was used to attract biotin-labeled capture antibodies to specific streptavidin-coated microelectrodes [32]. This electronic addressing system was applied to the detection of fluorescently labeled staphylococcal enterotoxin B and fluorescently labeled cholera toxin using an array microelectrode [32]. Each of the labeled toxins was concentrated from a mixture onto its specific antibody-coated electrode. In addition to the spatial positioning advantage offered by the electronic address-

ing method, the time required for antibody antigen binding was shortened due to the imposed potential at the electrode surface.

Another versatile and robust approach to immunochemical immobilization involves the use of peptide nucleic acids (PNAs) as a platform for attachment of an analyte derivative or capture antibody [33]. There are two advantages to this immobilization scheme. First, specific sequences of PNA can direct immunochemicals attached to complimentary oligonucleotides to specific locations on the array. Next, after the assay is complete, chemical disruption can be used to strip away the immunochemical and the same or different oligonucleotide-immunochemical conjugates can then be immobilized to the sensor surface.

Format innovations also include the use of reagentless immunoassay formats. One means to accomplish a reagentless format has been to tether the antigen analog onto the sensor surface next to the immobilized recognition element (e.g., antibody fragment) [34]. Signal transduction was accomplished using fluorescence resonance energy transfer (FRET). An additional advantage for the FRET format is that the observed fluorescence signal increases with increasing analyte concentration even though the assay is run in a competition format. This assay was characterized for the measurement of TNT over the concentration range of 0.1–60 mg/L.

One of the advantages of antibodies as recognition elements is their high affinities for target analytes. In many cases this results in very low detection limits for immunosensor assays. The disadvantage to this characteristic, however, is that the antigen is not easily released from the antibody after the measurement has been made. Several strategies have been used to design inexpensive biosensors around this characteristic. These strategies include the use of disposable sensors or sensing materials (e.g., glass or polystyrene beads) that can be detached from the detection instrument. An additional strategy involves the use of a flow cell configuration where the immunochemicals can be partially removed from a stationary sensor prior to the next measurement. Examples of disposable sensors have been reported using screen printed conductive ink electrodes to measure compounds such as PAHs [35]. Detection limits were in the low ng/mL range and antibody cross-reactivity was significant for 11 of the 16 priority PAHs examined. The assay was run in a competitive format and the alkaline phosphatase labeled secondary antibody resulted in the production of an amperommetrically detected enzyme product.

Another approach to this problem involves the use of polystyrene beads in a flow injection format. After analysis of coplanar PCBs using a competitive immunoassay format with HRP labeled competitor conjugate and fluorescent enzyme substrate, the beads were back flushed out of the detector chamber and another assay was run [36]. Although this recent report adapts the use of a replaceable solid phase immunoassay to a micromachined chip, this type of assay format has been previously reported in other instrument configurations [37].

Regeneration of the biosensor surface is another approach that has been used to solve the problem of tightly bound antibodies and antigens in non-disposable sensor surfaces. The use of PNAs to attach antibodies to sensor surfaces was discussed previously in this review [33]. Another approach involves the use of pepsin to release the antibody from an immobilized antigen without damaging the antigen-coated sensor [38]. In this competitive immunoassay system, TNT was selectively measured over a concentration range of 0.09–1000 ng/mL using surface plasmon resonance (SPR) as a means of detecting the binding of anti-TNT antibodies to the sensor surface. SPR was also reported as the signal transducer for an immunosensor for detection of bisphenol A [39]. The competition immunoassay was robust, stable and selective for the target analyte in surface water from several sources.

In addition to recent advances to antibody-based biosensors for chemical pollutants, advances have also been made for detecting environmentally relevant microorganisms. Challenges that face bacteria-detecting biosensors include detection limits and multi-species analysis. The use of an array biosensor approach has allowed the simultaneous detection of four species of shingella [40]. The array biosensor used a sandwich assay format. Detection limits for *S. disentariae* from washed chicken carcass were 4.0×10^4 cfu/mL and were 9.7×10^2 cfu/mL for *Camplobacter jejuni*.

An array immunosensor for detection of *Yersinia enterocolitica* has been reported using inkjet deposition of protein G which subsequently bound the monoclonal antibody [41]. Detection of the pathogen was accomplished using an imaging ellipsometry system. The calibration range for this assay was between 10^3 and 10^7 cfu/mL. Because the binding of the organism to the sensor surface could be directly detected, this immunosensor was considered to be a label-free format.

Immunosensor techniques can also be used to study antibody–microorganism interactions [42]. For example, a quartz crystal acoustic wave device was used to measure the binding of *Salmonella typhimurium* to the sensor surface by means of a more rigid somatic attachment or a more flexible flagellar attachment. The immunosensor was also selective for *S. typhimurium* in the presence of large concentrations of *Escherichia. coli*.

4. Cell-based biosensors

Cell-based biosensors for environmental applications can be organized according to cell type. For example, bacteria, yeast, algae and tissue culture cells. Although there are numerous examples of genetic modification to these cell types, genetically engineered bacteria (GEMs) are most often reported in cell-based biosensors [43]. Bacteria have been genetically engineered to construct gene fusions typically composed of a regulatory system (i.e., native promoter) linked to a reporter(s) genes. For these genetically modified microorganisms often referred to as 'biosensors' or 'bioreporters', the presence of an effector (nonspecific stressor or biochemically active compound or toxin) results in a cascade of events that produces some measurable response. Effectors for which bioreporters have been constructed include: non-specific stressors such as DNA damage, gamma radiation, heat shock, and oxidative stress; toxic metals such as cadmium, chromate, cobalt, copper, iron,

lead, mercury, nickel and zinc; organic environmental pollutants such as chlorinated aromatics, benzene derivatives, organic peroxides, trichloroethylene and PCBs; and compounds of biological importance such as nitrate, ammonia and antibiotics [40].

Genetically engineered microbial and cell-based biosensors show several advantages and limitations with respect to potential environmental applications. Microorganisms are in some ways quite robust sensing elements in that they are continually synthesizing complex systems of enzymes, cofactors and nucleic acids. Once constructed, they are self replicating and many require only the effector to elicit a response. Bioreporter microorganisms also show the potential to be interfaced to a wide range of transducers including optical, electrochemical, piezoelectric and surface plasmon resonance. Limitations primarily involve the maintenance of their environment (i.e., nutrients, O_2, pH, ionic strength, etc.) and the time required for a response. For systems that require expression of proteins responsible for the transduction signal, the time frame can be on the order of hours. In many cases, there is an analyte concentration window where the organism will respond. Concentrations below this window will not elicit a response and concentrations above will be toxic to the microorganism also resulting in a negative response.

Recent advances in reporter microorganisms have involved novel fusions of a wide range of promoters with conveniently measured reporters, as well as the construction of unique sensing platforms that can be used to study individual organisms as well as population responses. One of the classes of environmental pollutants that are well suited to bioreporter microorganisms are the volatile organic compounds (VOCs). Several early warning systems for VOCs using GEM systems have been described. In one example, a benzene sensitive promoter (ps) was linked to the production of green fluorescent protein (GFP) [44]. This reporter was responsive to 10 benzene derivatives. Aromatic hydrocarbons were measured in the 0.1 mM concentration range using a fluorescence microplate reader. In another biomonitoring configuration, the aromatic hydrocarbon-sensitive xys promoter of the TOL operon was fused to the β-galactosidase and alkaline phosphatase reporter genes [45]. The presence of aromatic hydrocarbons such as xylene and toluene were measured using the catalysis of non-active enzyme substrates to electrochemically active products. Eight reaction chambers could simultaneously screen environmental samples for the presence of toluene or xylene isomers in the μM concentration range. In addition, benzene and toluene vapors introduced through a side arm flask were measured using this bioassay system.

In another study, the TOL containing plasmid was fused with the gene for firefly luceferase and inserted into *E. coli* [46]. This transformed organism was characterized for environmental monitoring of benzene derivatives. The observed concentration response curves were biphasic with the bioluminescence response increasing between 0 and 0.5 ppm then decreasing between 0.5 and 5.0 ppm. The time response of this system for benzene increased between 2 and 8 h, and then decreased out to 12 h after the initial exposure.

Unique assay platforms have also been reported for GEM systems. For example, a bioreporter strain of *E. coli* that produced GFP in response to arsenite and antimonite was used to demonstrate a microfluidics platform [47]. Comparison of this system to a standard fluorescence curette platform indicated that mixing and analysis using the centrifugal microflow system decreased the time required for the assay and increased the reproducibility. Another unique assay platform that has been demonstrated for an *E. coli* strain that is responsive to Hg(II) involved immobilization of individual bacteria into microwells on the face of an imaging fiber [48]. Locations containing cells in the microwell fiber array were identified by the presence of enhanced cyan-fluorescent protein and the response to Hg(II) was measured using the enhanced production of β-galactosidase that catalyzed the formation of a fluorescent enzyme product optically distinguishable from the cyan-fluorescent protein. Both individual cells and cell average responses were used to measure Hg(II) in the 0.1–5 μM concentration range.

In addition to responding to specific compounds, ions or classes of related compounds, microorganisms have also been engineered to respond to cytotoxic and genotoxic compounds using a variety of mechanisms. In one example, *E. coli* carried a plasmid where the DNA damage inducible rec A promoter was linked to the GFP reporter [49]. This bioreporter strain responded to the genotoxic agents mitomycin C, *N*-methyl-*N*′-nitroguanidine and nalidixic acid. Relative fluorescence of the inducible GFP occurred within a time frame of about 2 h. In another system, several strains of recombinant bioluminescent bacteria, responsive to superoxide (damage), hydrogen peroxide and DNA damage were each used in a flow through bioreactor [49]. The bioreactor allowed cell densities to remain the same while the luminescence responses of various strains sensitive to paraquat, mitomycin C and hydrogen peroxide were measured at detection limits of 0.05, 0.05 and 10 mg/L, respectively.

One of the challenges in measuring genotoxicity using GEM bioreceptors involves the threshold for cytotoxicity which is often slightly above the concentration required for a genotoxic response. A recently described system for genotoxicity using genetically modified yeast that express GFP in response to genotoxins reported both genotoxicity (using GFP) and cytotoxicity (using cell density) [50]. The authors have reported responses to a broad range of compounds. This system has also been used to assess the toxicity and genotoxicity of environmental samples using a field portable instrument [51]. In another approach to simultaneous monitoring of genotoxicity and cytotoxicity, a bioreporter was constructed using *Salmonella choleraesuis* that detected genotoxins using bioluminescence and cytotoxins using the production of GFP [52]. This bioreporter could distinguish between genotoxins such as mitomycin C and cytotoxins such as aureomycin. The assay system was also demonstrated using contaminated environmental samples from different locations in the Punjab river basin in Pakistan.

Recent advances in bacterial luminescence assays for cytotoxicity primarily involve linking them to specific environmental applications. One example of this type of application is an automated continuous toxicity monitoring system using the genetically engineered fresh water bacterium *Janthinobactrium Ividum* YH9-RC that is sensitive to heavy metals as well as a number of toxic organic compounds [53]. For this system, cell

cultures were lyophilized in a series of 384-well plates which were automatically reconstituted with waste water and the bacterial response recorded allowing the determination of toxicity spikes in a sample stream.

In addition to organic pollutants likely to result from point sources, GEMs have also been reported to measure pollutants resulting from agricultural run-off. A nitrate bioreporter from *E. coli* was recently reported where the nitrate reductase operon (pnar) was fused to the GFP reporter (gfp) [54]. This reporter bacterium was used to measure nitrate in environmental waters and was free from interferences from compounds such as dimethyl sulfoxide, trimethyl amine-*N*-oxide fumarate, and azide. In another study, several strains of reporter bacteria were used to assess the bioavailability of heavy metals in soil samples containing mixed organic waste [55]. By using reporter strains sensitive to general toxicity as well as those specific for heavy metals, the bioavailability of metal ions could be better evaluated.

Unmodified bacteria have also been used as biomarkers and bioindicators of generalized or compound-specific toxicity. For example, activity of the enzyme delta-aminolevulinate dehydratase is a human biomarker for lead exposure [56]. This enzyme has also been used as an indicator for lead bioavailability in *Pseudomonas pudita* where it has been used to measure lead exposure in the μM concentration range [56]. *P. pudita* has also been recently used as a bioreporter for benzene [57]. Bacterial cells were immobilized to a dissolved oxygen probe and used to measure benzene vapors that had been trapped on a solid phase sorbant and released into phosphate buffer. In another example, *E. coli* cells that were immobilized onto a gold sensor by self assembly using cysteine terminated synthetic oligonucleotides showed a decrease in cell viability when exposed to phenol [58]. The decrease in cell viability was measured by a change in plasmon resonance angle and yielded a detection limit for phenol of 5 mM. A piezoelectric quartz crystal was also used to measure the growth of bacteria isolated from a costal lagoon [59]. This system was used to measure Cu(II) which increased cell growth at concentrations below 18.8 μg/L and inhibited cell growth at concentrations above 25.0 μg/L.

Algae have also been used in cell-based biosensors and bioassays to measure several classes of environmental pollutants. In one example, the cyanobacterium *Spirulina subsalsa* was immobilized to an oxygen probe and placed in a flow cell configuration. This algal biosensor was reversibly responsive to heavy metals, triazine herbicides and carbamate insecticides [60].

The use of a wild type and analyte resistant genotype of *Dictysphaerium chorelloides* were used to confer sensitivity and selectivity in the measurement of TNT [61]. Inhibition of the chlorophyll A fluorescence of photosystem (PSII) after a three min exposure was used as the biological signal to measure TNT over a concentration range of 0.5–31.3 mg/L. In another algal biosensor configuration, vapors of formaldehyde (0.05–1 ppm) and methanol (200–1000 ppm) were measured using a 4×3 array of *Klebsormidium* and *Chlorella* algae immobilized to a cellulose ester membrane [62]. Detection of Chlorophyll A fluorescence was measured using a specialized fluorimeter and CCD-camera.

A wide range of cell-based biosensor and bioanalytical assay systems have been employed for general characterization of ground water and waste water toxicity as well as for use as biological early warning systems (BEWS) in water treatment facilities. Three basic analyses where biosensors have made contributions include biological oxygen demand, toxicity analysis and detection of pathogenic organisms [63]. Biosensor applications for measurement of biological oxygen demand have been extensively reviewed [2]. A recent report suggests the use of a BOD biosensor to monitor and improve waste water management practices [64].

There are several features that are required for toxicity-based early warning systems that are based on metabolic inhibition. These bioassays should be simple (requiring less than 1 day training), cost-effective (compared to standard analytical methods), and respond to a wide range of compounds at concentrations relevant to their regulatory limits. In addition to commercially available systems that have been well characterized and have been in use for a number of years such as Microtox™ and ToxAlert™, there have been a number of recent reports describing bioassay systems that improve upon various functional aspects of these assays. For example, the Cellsense biosensor system uses *E. coli* that is immobilized to an electrochemical transducer [65]. This system can be used in a continuous operational mode and has been characterized using a number of toxic industrial compounds with environmental matrices. Another recent study has compared the relative responsiveness of several bioassays based both on bacterial metabolism and catabolic activity [66]. *Vibrio fischeri, Pseudomonas fluorescens* 10568, *E. coli* HB101, *P. putida* TVA8 and *E. coli* DH5α were evaluated using ground water samples contaminated with a range of chlorinated hydrocarbons. Each bioreporter strain showed different sensitivity profiles toward the differentially contaminated ground water samples. Due to the complementary responses of the bioreporters tested, the authors suggested the use of a battery of tests for environmental application. In addition, this study outlined advantages for the use of these assays in monitoring bioremediation processes where the relative composition of polluted environmental media has been previously determined.

One of the unexpected characteristics of bacterial bioreporter assays involves the synergistic effects observed from exposures to several compounds. For example, in studies using *V. fischeri* as a bioreporter to measure the toxicity of environmental ground water, six volatile organic compounds showed a synergistic effect resulting in EC_{50} values that were an order of magnitude lower than expected from additive effects of each of the individual compounds alone [67]. Although this synergistic effect may not apply in the case of potential human exposure, the relevance with respect to microbial ecology is compelling. Although most cell-based bioassays for environmental applications use bacteria, yeast or algae, there have been several recent reports that describe the use of mammalian tissue culture cells. These bioassay systems have, in some cases, been linked to receptor binding assays such as those using the aryl hydrocarbon receptor (AhR) or complex cellular responses. For example, binding of dioxin-like compounds to the AhR in tis-

sue culture cells has been measured using the dioxin responsive element sensing via secreted alkaline phosphatase (DRESSA) system [68]. Recent improvements in assay time and sensitivity to compounds such as 3-methylchloranthrene, benzo(a)pyrene and β-naphthoflavone have increased the usefulness of this assay for environmental applications as well as increased the feasibility for potential interface to optical or electrochemical transducers. In another example, human umbilical vein endothelial cells (HUVECs) have been grown to confluency on a modified cellulose triacetate membrane and plated over an ion selective electrode [69]. Exposure of this biosensor to the ion permeability enhancing toxin histamine, resulted in a concentration-dependant electrochemical response.

5. DNA biosensors

Due to their wide range of physical, chemical and biological activities, nucleic acids have been incorporated into a wide range of biosensors and bioanalytical assays, many of which show the potential for adaptation to environmental applications. More specifically, as previously mentioned in this review DNA has been used to measure Pb^{2+} by virtue of its catalytic activity [25,26]. DNA and PNA have also been used to link immunochemicals to specific locations in DNA chip arrays by means of hybridization of complementary oligonucleotides [33].

In addition to these genetically unrelated uses of DNA for biosensors, several biosensors and bioassays have been reported for the detection of chemically-induced DNA damage. There is also an ongoing effort in the area of biosensor technology for measuring DNA hybridization prerequisite for genetic identification of pathogenic microorganisms.

The measurement of DNA damage using electrochemical biosensors has been demonstrated using the direct measurement of oxidation–reduction properties of the bases [70,71] or indirectly using electrochemical probes [72]. These biosensors have been used to measure toxic aromatic amines [71], oxidative damage [72,73], and bioactivated benzo(a)pyrene [74]. DNA damage has also been measured using fluorescence-based biosensors and bioanalytical techniques. Time resolved fluorescence measurements were used to detect radiation-induced changes in DNA unwinding behavior from DNA isolated from white blood cells [75]. In another recent report, DNA adducts of benzo(a)pvrene were measured using low temperature fluorescence on a gold biosensor chip [76]. The adducts were captured using structure-specific monoclonal antibodies that were immobilized using a chemical protein linker and recombinant protein A. Detection limits for DNA adducts were in the low femtomole range.

Changes in melting-annealing behavior that were observed in real-time using a double strand-selective fluorescent indicator dye have also been used to measure DNA damage induced by radiation and chemical mutagens such as styrene oxide, glutaraldehyde, and benzo(a)pyrene [77,78]. This rapid screening assay was sensitive to various forms of DNA damage including strand breaks, crosslinks and adduct formation.

DNA hybridization microarrays have been suggested as a platform for the parallel detection of multiple pathogenic microorganisms relevant to both biodefense and environmental contamination applications [79]. Although a wide variety of biosensors and bioanalytical test kit assays have been reported to detect a limited variety of organisms, these authors [79] suggest that a significant number of genetic identifiers or virulent factors could be targeted by this approach. The technology required for this type of application would require that biosensors be rapid, sensitive, and compatible with commercial development. Examples of this approach have been demonstrated for the food industry. DNA microarray techniques have been reported for the simultaneous detection of food borne pathogens and their virulence factors [80]. More specifically, this DNA hybridization array technique allowed for the detection and identification of multiple species of *Campylobacter* and *Listeria* as well as a wide range of staphylococcal enterotoxins and *C. perfringens* toxins.

Advances in the development of hybridization biosensors have also included a visual DNA chip for detection of hepatitis virus [81], hybridization using lead labeled oligonucleotides detected by anodic stripping voltammetry [82], optical detection of hybridization using gold nanoparticles [83], and electrochemical detection of DNA hybridization using silver precipitation on gold nanoparticle-labeled oligonucleotides [84,85]. Although these biosensors and array techniques were not specifically developed for environmental applications, this type of technology is prerequisite for development of DNA-based biosensors for environmental applications.

6. Receptor-based biosensors

Receptor-based biosensor systems have the inherent advantage in that any detrimental environmental pollutant that will bind to the receptor at physiologically relevant concentrations can potentially be measured. Thus, these systems can be used to screen for a wide range structurally divers pollutants with a similar mechanism of toxicity.

Recent advances for receptor-based biosensors for environmental applications have focused on the human estrogen receptor-α. Development of these assay systems has primarily resulted from a concern over the adverse effects of endocrine disruptors on human health. For example, an endocrine receptor-based assay has been developed using the BIAcore (plasmon resonance) sensor platform [86]. This system was characterized for a wide range of estrogenic and non-estrogenic compounds. The assay was also configured as an enzyme-linked microplate assay similar to an ELISA format.

The estrogen receptor has also been incorporated into an electrochemical biosensor format based on impedance measurements [87]. This biosensor was used to measure estrogen, bisphenol A and geistein. Another bioanalytical approach to measuring estrogenic and estrogen disrupting compounds used genetically engineered ligand-inducible luciferase-expressing cell lines [88]. These cells could be induced to produce a concentration-dependent luminescent response using a range of natural and synthetic estrogenic compounds. In addition to estrogen receptor-based biosensors, a number of antibody-based biosensors have been recently reported for detection of estrogenic compounds. These systems include plasmon resonance-based (BIAcore) immunosensors for bisphenol A [89] and 4-

nonylphenols [90], Both RIANA and AWACSS continuous in-line water monitoring biosensors discussed in the immunosensor section of this review have also been configured to measure a number of estrogenic and endocrine disrupting compounds [18,19,31].

7. Future directions

Biosensors for potential environmental applications continue to show advances in areas such as genetic modification of enzymes and microorganisms, improvement of recognition element immobilization and sensor interfaces, and introduction of improved operational formats and unique environmental applications. The use of genetically modified AChE in biosensors has significantly increased their sensitivity to inhibition by OP pesticides [6–8]. Furthermore, genetic modification shows the potential for selection of enzyme variants that are specific for a range of individual compounds.

Novel gene fusions have resulted in more sensitive and versatile reporters such as GFP and show the potential for construction of a battery of organisms that respond to a wide range of physical and chemical stressors using a single detection platform. One area where continued progress could yield significant advances for environmental applications would be to better characterize bioreporter organisms as surrogates for human exposure.

Better methods for immobilization of enzymes and antibodies to sensor surfaces continue to increase the robustness and improve prospects for commercialization of biosensors for environmental applications. Future advances in immobilization will likely focus on directing biorecognition elements to addressable locations on micro or nano-sensor arrays using strategies such as hybridization of sequence-specific PNAs [33]. The ability to construct arrays of enzymes, antibodies or oligonucleotides will likely allow current multianalyte detection of several compounds or microorganisms to be expanded to accommodate the analysis of perhaps hundreds or thousands of separate compounds. One of the challenges that must be met for this type of system would be the development of parallel computational methods to convert electronic responses for each analyte into meaningful concentration data. In this respect progress has been reported for automated sensor systems that operate in environmental settings [31].

A biosensor approach toward measuring genetic damage has involved the detection of chemically-induced damage to surrogate DNA [70]. One of the challenges for this area will be the development of environmental applications related to ecosystem and human exposure to genotoxins. This would require the isolation and analysis of DNA after a suspected exposure of the organism to the genotoxic substance. This type of application would also require both high sensitivity to the extent of DNA damage and to the amount of DNA required for the analysis.

The development of biosensors receptive to biochemical responses are also a trend where significant advances are likely to be made in the future. For example, a range of receptor-based biosensors that are receptive to estrogenic and endocrine disrupting compounds have been reported [86–88]. The challenge in this case will be to calibrate the biosensor response to the risk of adverse biological effect rather than simply receptor binding affinity.

Biosensor techniques for potential environmental applications have continued to show sustained advances in a wide range of areas. It is also likely that these advances will play an important role in the development of biosensor systems for the environmental market. Nevertheless, until biosensors achieve operational characteristics similar to the simple pH electrode in terms of durability, selectivity, extended concentration range, and resistance to biofouling, they will likely continue to experience significant obstacles to widespread acceptance and use for environmental monitoring.

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