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Research Article

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Evaluation of Whole Cell Biosensors for Usability in On-site Detection of Two Major Classes of Antibiotics in Agricultural Soil and Water

Abstract

Human health is heavily influenced by the environment. In recent years, the contamination of soil and water by antibiotics has become a major public health issue. This is because of the selective pressure from antibiotics in the environment that results in the proliferation of antibiotic-resistant bacteria. A major contributor to the emergence of antibiotic resistance is the indiscriminate use of antibiotics in the agriculture and medical industry, followed by insufficient antibiotic-removal treatment of wastewater from these industries, resulting in the antibiotic accumulation in the environment. Limiting the use of antibiotics must be followed by extensive surveillance to track antibiotic residue levels in agricultural soil and water samples. In recent years, there has been a growing interest in the use of whole cell biosensors to monitor levels of antibiotics in agricultural samples; this is because whole cell biosensors are portable, cheaper, and simpler to operate and interpret compared to traditional methods of antibiotic detection. This review article compares the potential of existing β-lactam and tetracycline whole cell biosensors for on-site analysis of agricultural soil and water samples based on practicality, performance, robustness, and range of detection. Despite the lack of data regarding the performance of these biosensors under varying pH and temperature conditions, this review weighs the benefits and drawbacks of each biosensor to determine a promising candidate for use in on-site detection of β-lactams and tetracyclines. Of the β-lactam biosensors examined, only a *Bacillus subtilis*-based biosensor was able to detect β-lactams in water samples with high sensitivity and specificity while producing a strong and stable signal. However, this biosensor was not tested in soil samples, has a relatively long response time, and requires a spectrophotometer to view the signal. Engineering the reporter gene to produce a colorimetric signal will increase its potential in on-site detection. Of the tetracycline biosensors examined, a compact paper strip biosensor was found to be sensitive and highly practical when tested in both soil and water samples. Thus, we determined it to be the best candidate for on-site detection. This biosensor, however, also suffers from relatively lengthy response times. The realization of these biosensors as tools for antibiotic surveillance depends on further experimentation using on-site samples.

Introduction

The rapid emergence of antibiotic resistant bacteria is the greatest imminent threat to global health[1]. Antibiotic resistance is a naturally occurring process that is greatly expedited by antibiotic use, as antibiotics kill susceptible bacteria and allow resistant bacteria to survive and replicate. The pervasive overuse of antibiotics in agriculture and medicine are two major contributors to antibiotic resistance[1,2]. Conventional wastewater treatment cannot entirely remove antibiotics from sewage produced by the medical industry. Furthermore, the lack of effective antibiotic-removal treatment in agricultural systems gives rise to antibiotic accumulation in agricultural water and soil[2].

To monitor and treat antibiotic overuse, it is necessary to develop methods to support widespread and continuous surveillance of antibiotic levels in agricultural soil and water. Currently, several antibiotic detection methods are available. Traditional chemical assays use liquid chromatography and mass spectrometry to extract antibiotics from a sample[3]. These methods can be extremely sensitive, being able to detect antibiotics at a concentration as low as 0.05 ng/mL[4]. However, traditional methods require expensive equipment, experienced technicians, and complex, lengthy processing, making them impractical for on-site antibiotic detection in agricultural soils and water[4]. Conventional biosensors that use aptamers or antibodies as antibiotic recognition elements may be more portable, but are expensive and only stay sensitive for a small range of ion concentration, pH, and temperature, thus limiting their suitability for on-site detection of antibiotics[4].

Whole cell biosensors have been developed to address the limitations of chemical-analytical detection methods and conventional biosensors. A whole cell biosensor reprograms existing signalling pathways in living cells to respond to the critical levels of antibiotics by producing a visible output. Whole cell biosensors for antibiotics are usually constructed via fusing a reporter gene to a promoter, which acts as an antibiotic recognition element[5]. Whole cell biosensors are sensitive, specific, cheap, and portable. Furthermore, they produce easily interpretable results rapidly, do not require specialized equipment, and accurately report the concentration of bioavailable antibiotics[5]. These characteristics make whole cell biosensors an appealing tool for on-site analysis of agricultural soil and water samples.

This review aims to evaluate existing whole cell biosensors for two representative classes of antibiotics commonly used in agriculture and medicine: tetracyclines and β-lactams. The whole cell biosensors' usability in on-site analysis of agricultural soil and water samples will be examined. This review will consider the biosensors' [1] practicality; [2] performance, using metrics such as specificity, signal stability, and response time; [3] response format (responding in a dose-dependent manner is preferred over binary classification, since a quantitative response would provide useful information for subsequent antibiotic removal and sample treatment in these soil and water); [4] range of detection (they must be sensitive enough to detect the range of antibiotic concentration predicted to select for resistance bacteria (β-lactam: 0.25-4 ng/mL, tetracycline: 1-16 ng/mL)[6]); and [5] robustness (whether they retain their sensitivity in pH and temperature ranges presented by farm water and soil samples). While countries around the world would have varying pH, temperature range, and antibiotic concentration values in soil and water[7,8,9,10], they do not deviate greatly from the values obtained in Canada, which are shown in Table 1[11,12,13,14,15,16,17].

Table 1. pH, temperature, and antibiotic concentrations in agricultural soil and water samples in Canada

	pH	temperature (°C)	[tetracycline] (ng/mL)	[β-lactam] (ng/mL)
water	6.5-8.5 [11]	0-25 [12]	0-400 [13]	0-42.2 [14]
soil	5.5-8.0 [15]	0-20 [16]	0-249 [13]	0-6720 [17]

Biosensors for β-lactams

β-lactams are one of the most prescribed antibiotic classes and the most commonly used bactericidal agent in agriculture[18]. They are bactericidal agents that kill bacteria by inhibiting the production of peptidoglycans in bacterial cell walls. Bacterial β-lactam resistance arises mainly based on the synthesis of β-lactamase enzyme, which cleaves the β-lactam ring and inactivates the antibiotic. A number of whole-cell sensors are available for detection of β-lactams. Lautenschläger et. al. developed a *Bacillus subtilis (B. subtilis)*-based whole cell biosensor by fusing the promoter P*blaZ* with the luciferase reporter gene *luxABCDE* downstream of the β-lactam-activated BlaR1/BlaI signalling pathway[19].

Without the presence of β-lactam, BlaI represses the *blaZ* pathway by binding to the promoter P*blaZ*[20]. As β-lactam enters the whole cell biosensor, it binds to BlaR1 and activates BlaR1's proteolytic activity[20]. BlaR1 degrades BlaI and frees the target promoter, allowing the expression of the downstream luciferase gene, which emits bioluminescence. The functionality of this sensor was tested on ten β-lactam derivatives, representing all four subclasses of β-lactams—penicillins, cephalosporins, monobactams, and carbapenems, as well as on the cyclic polypeptide antibiotic bacitracin as a negative control[19]. The sensor detected all ten β-lactam derivatives with high specificity in *Streptomyces* soil isolates and water samples and had a lower detection limit of 1 ng/mL[19]. *Streptomyces* are known to produce a large variety of antimicrobial compounds, among them β-lactams[19]. The *Streptomyces* soil isolates were screened by the biosensor for β-lactam production through a modified disk diffusion assay[19]. β-lactam derivatives induced a luciferase signal within two hours that remained stable for several hours, while the control bacitracin did not induce a luciferase signal[19].

While the luciferase signal needs to be viewed using a spectrophotometer, decreasing the portability of this assay, the development of sensitive and robust hand-held luminometers would solve this problem. Alternatively, changing the reporter to β-galactosidase and using a chromogenic enzyme substrate would allow the biosensor to produce a signal that is visible to the eye. β-galactosidase reporter genes have been incorporated in whole-cell biosensors for on-site detection of bacitracin, another type of antibiotic, for as low as 49.3 ng/mL[21]. Its application in the detection of β-lactams or other types of antibiotics is a promising direction of research. This study did not test the functionality of the biosensor in varying pH and temperature conditions encountered in the field, but the authors claim that the sensor can be used to analyse weakly acidic samples[19]. B. subtilis can grow in pH 4 to 9.5, and its optimal temperature range is 25 to 35 °C; these numbers can serve as a rough estimate for the pH and temperature range of the biosensor[22]. This *B. subtilis*-based biosensor detects β-lactam derivatives with adequate sensitivity and specificity in water samples and bacterial soil isolates, as its lower detection limit falls within the range of antibiotic concentration predicted to select for resistance. It is likely to function within the pH range of most agricultural samples, and is unlikely to require temperature control, suggesting that this sensor is robust enough to be used for on-site monitoring. However, the response time was relatively slow at two hours compared to other biosensors discussed in this review, and the luciferase signal must be detected using specialized equipment. The biosensor should be tested in various pH and temperature conditions, as well as soil samples, after making a β-galactosidase reporter gene substitution to produce a colorimetric output.

Valtonen et al. developed an *Escherichia coli (E. coli)* sensor with a luciferase reporter under the control of the β-lactam-responsive element *ampR/ampC*[23]. The inducible β-lactamase promoter *ampC* is under transcriptional control of a regulator encoded by *ampR*. The presence of β-lactam leads to breakdown of microbial cell wall murein structures. These murein products bind to *ampR*, and in turn *ampR* activates the *ampC promoter* and its downstream gene, the luciferase[23]. The biosensor was able to detect six β-lactam derivatives (ampicillin, piperacillin, imipenem, cephapirin, cefoxitin, and oxacillin) with high specificity. The biosensor had a detection range of 2.5 ng/mL to 250 µg/mL.

The signal was generated in two hours but was unstable, especially when the concentration of β-lactam was low. As with the *B. subtilis*-based sensor, acquiring a suitable handheld luminometer or the replacement of the luciferase reporter with β-galactosidase reporter would remove the requirement for a spectrophotometer. The authors only tested the biosensor in laboratory conditions and did not analyse soil or water samples. For an approximation of the pH and temperature range of the biosensor, *E. coli* can grow in pH 6.3 to 7.8 and a temperature of 19.3 to 41 °C[24]. Under these conditions, *E. coli* can survive in soil and water for approximately 90 days[24]. The biosensor is therefore likely to be robust enough to be used for on-site monitoring. The biosensor can be freeze-dried and used instantly after being rehydrated, without any growth step, eliminating the laboratory cultivation period prior to use[23]. Further experimentation is needed to determine the sensor's functionality for soil and water samples. The slow and unstable signal output is a major disadvantage of this biosensor.

Kumar et. al developed a *Pseudomonas aeruginosa (P. aeruginosa)*-based potentiometric biosensor for one derivative of β-lactam antibiotics, cephalosporin, with a detection range of 40 to 400 µg/mL[25]. A layer of biosensor was immobilized to a cellulose acetate membrane, and permeabilized with lysozyme[25]. The lysozyme-permeabilized biosensor cells hydrolyse a β-lactam ring of cephalosporin, producing cephalosporin acid[25]. The protons generated in this reaction are detected by pH electrodes[25]. The study did not test whether this biosensor can be used to analyse soil samples. The low sensitivity of this biosensor is another major drawback, as the lower detection limit of the biosensor would be too high to detect cephalosporin in most agricultural water samples. Furthermore, the biosensor is the least robust of all those presented in this review; to achieve maximum sensitivity, the temperature of the biosensor must be kept at 35 to 40 °C, and the pH must be kept at 7. Another limitation of this biosensor is that it can only detect one antibiotic derivative. Despite these drawbacks, the biosensor's strength is in its short response time: a strong and extremely stable signal is produced within three minutes, giving the biosensor the potential to be used in real-time monitoring of cephalosporin levels. To improve its range of usage, water evaporation methods can be used to concentrate the sample, meeting the detection limit. However, the evaporation process takes away the advantage of this sensor: its short response time. The low sensitivity coupled with strict pH and temperature requirements of this biosensor impede its practical application in on-site monitoring.

Of the three of β-lactam biosensors presented in this review, only the *B. subtilis*-based whole cell biosensor was tested using water samples, and only the *P. aeruginosa*-based biosensor was tested for functionality in varying pH and temperature conditions. None of the biosensors was tested on soil samples directly. With incomplete data on the robustness and performance of all three biosensors in water and soil samples, it is difficult to make a definitive judgement with regards to which biosensor is most suitable for on-site detection of β-lactams. The *B. subtilis*- and *E. coli*-based biosensors were able to detect β-lactams with high sensitivity and specificity, as its detection range covers the range of antibiotic concentration that selects for resistance[6]. They could be modified slightly to produce a colorimetric output, which is convenient for on-site monitoring in a low-tech environment. Both biosensors had relatively longer response time compared to the *P. aeruginosa* biosensor, but only the *B. subtilis*-based whole cell biosensor produced a stable signal. While the *P. aeruginosa* biosensor had a significantly shorter response time, its low sensitivity makes it unsuitable for analysis of agricultural water samples and its diffusion-dependent construct makes it unsuitable for soil sample analysis. Based on the available data, the *B. subtilis*-based biosensor is the best candidate for on-site detection of β-lactams in agricultural soil and water.

Table 2. Properties of three β-lactam biosensors

Author name	Lautenschläger et. al	Valtonen et. al	Kumar et. al
Whole cell species	*Bacillus subtilis*	*Escherichia coli*	*Pseudomonas aeruginosa*
Output	Fluorescence	Fluorescence	Electrochemical (proton)
pH	4.5-9 (estimated)	6.3-7.8 (estimated)	7.0 optimal
Temp (°C)	25-35 (estimated)	19.3-41 (estimated)	35 to 40 optimal
Detection Range	1 to 300 ng/mL	2.5 ng/mL to 500 µg/mL	40 to 400 µg/ml
Antibiotic subclasses detectable	10 derivatives	6 derivatives	1 derivative
Signal start up time and stability	2 hours, stable for several hours	2 hours, unstable after one hour with low concentration	3 minutes, stable for 8 days
Tested in	*Streptomyces* soil isolates and water, has potential in milk (slightly acidic)	Culture	Phosphate buffer + medium

Biosensors for tetracyclines

Tetracyclines are a class of bacteriostatic antibiotics that inhibit bacterial growth by inhibiting protein synthesis[26]. Tetracyclines are the most frequently used antibiotics in agriculture because they can be synthesized with high purity and are cheap to produce[26]. However, they are not absorbed well by animals' intestines and therefore contribute to significant long-term contamination of groundwater through animal waste[26]. As a result, the prevalence of tetracycline resistance is very high, reaching 67% for E. coli in several European countries[27].

Various whole cell tetracycline biosensors are available. Hansen and Sorenson developed three *E. coli*-based biosensors by fusing the tetracycline inducible *tet* promoter with different reporter genes: *lacZYA*, which encodes β-galactosidase, Green Fluorescent Protein (GFP), and *luxCDABE* luciferase[28]. All three biosensors were quite sensitive, being able to detect as low as 10 ng/mL of the tetracycline derivative oxytetracycline[28]. The *lacZYA* biosensor had a relatively short response time of 50 minutes and produced a stable signal that lasted for 5 days, while GFP and *luxCDABE* biosensors had longer response times of three hours[28]. The authors did not report the stability of the GFP and *luxCDABE* signals. Unlike the GFP and *luxCDABE* biosensors, the *lacZYA* biosensor produces a colorimetric output and does not require specialized equipment. The authors did not analyze soil samples and only tested the biosensors under laboratory conditions. They noted that the GFP and *luxCDABE* biosensors could not function well at temperatures higher than 30 °C[28]. E. coli can grow in pH 6.3 to 7.8 and a temperature of 19.3 to 41 °C[24], but this does not mean that the biosensor can function well within this whole range. The most promising biosensor, the *lacZYA* biosensor, should be tested using soil and water samples under varying pH and temperature conditions.

Ma et al. developed a paper strip *E. coli*-based whole cell biosensor for tetracycline by liquid-drying *E. coli* cells that expressed the *lacZ* reporter gene under the control tetracycline-inducible *tet* promoter onto strips of filter paper[3]. The paper strips were photographed and analysed digitally to determine quantitatively the color intensity of the blue area of the paper[3]. The biosensor could detect six tetracycline compounds (tetracycline, oxytetracycline, chlorotetracycline, deoxytetracycline, minocycline, and methacycline) with high specificity[3]. The authors claimed that the biosensor is more robust than typical whole cell biosensors, having the ability to accommodate a wider range of pH, temperatures, ionic strengths, and presence of other contaminants such as fertilizers, because it is a self-contained paper strip[3]. However, the authors did not support most of these claims with experimental data. The biosensor is more sensitive than the biosensor by Hansen and Sorenson, with a detection limit of 7.5 ng/mL in water and in soil samples[3]. Samples were collected from tetracycline-contaminated soils, mixed with EDTA solutions acting as sensitizers, sonicated with ultrasound, centrifuged, and filtered[3]. The processed soil extracts were mixed with Lysogeny broth and tested using the paper strip biosensor[3]. If the soil extract processing can be simplified, then the paper strip format makes the biosensor highly portable and convenient, removing any requirement for well-trained personnel. The biosensor had a longer response time than the biosensor by Hansen and Sorenson, generating a clear signal 90 minutes after incubation with low concentrations of tetracycline[3]. However, the colorimetric output, relatively ease of use, and compatibility with soil samples makes this biosensor effective for on-site monitoring of tetracyclines. Additional testing is needed to validate the specificity and robustness of this biosensor. Significant attention should also focus on simplifying the soil extract processing to highlight the advantage of this biosensor: portability and ease of use.

Bahl et. al developed an *E. coli* whole cell biosensor by fusing the tetracycline-induced promoter *tet* with the GFP reporter gene[4]. This biosensor was able to achieve the lowest detection limit among all tetracycline biosensors being compared in this review: 5 ng/mL[4]. The flow cytometry was used to detect and quantify four tetracycline compounds[4]. While the concept of using flow cytometry for antibiotic detection is certainly novel and promising, the complicated design and bulkiness of flow cytometers limits the feasibility of implementing such a method in the field for on-site detection of tetracycline. Moreover, this method requires cells to be removed from the detection site and into the flow cytometer; fluorescent signals might be lost during this transportation[4]. Development of miniaturized flow cytometry devices would potentially allow this sensitive method to be used on site. Furthermore, the experimenters did not test the ability of this biosensor to analyse environmental water and soil samples. Analysis of soil samples using flow cytometry would require the separation of the biosensor bacteria from soil particulates, further reducing the efficiency of this method[29].

For the three tetracycline whole cell biosensors discussed in this review, although all their lower detection limit falls within the range of concentration that selects for resistant bacteria, there is still a trade-off between sensitivity and adaptability. The flow cytometry method developed by Bahl et. al is slightly more sensitive than the others, but far less practical for on-site monitoring. The highly portable paper strip biosensor is the most practical, has the greatest potential, and has a greater sensitivity than the biosensor produced by Hansen and Sorenson. Regrettably, of the three biosensors presented in this review, only the paper strip biosensor was tested using soil and water samples, and none of the studies thoroughly tested the ability of the biosensors to accommodate pH and temperature conditions encountered in the field.

Table 3. Properties of three tetracycline biosensors

Author name	Hansen and Sorenson	Ma et al.	Bahl et al.
Whole cell species	*Escherichia coli*	*Escherichia coli*	*Escherichia coli*
pH	6.3-7.8	6.3-7.8	6.3-7.8
Temperature	19.3-41 °C	19.3-41 °C	19.3-41 °C
Output	Fluorescence or colorimetric	Colorimetric	Fluorescence
Detection range	10 ng/mL - 10 µg/ml	7 ng/mL - 10 µg/ml	5 ng/mL-16 µg /mL
Antibiotic subclasses detectable	1 derivative	6 derivatives	4 derivatives
Signal start up time and stability	50 minutes, stable for 5 days	1.5 hours, stability unknown	unknown
Tested in	culture	Soil and water	water

Reporter genes for on-site detection

Three different reporter genes have been employed in the construction of whole cell biosensor in this review: luciferase, GFP, and β-galactosidase. β-galactosidase hydrolyzes lactose, and in colorimetric assays, lactose analogues (X-gal or ONPG) become colored after hydrolysis[30].

As production of β-galactosidase increases, the intensity of the color increases in the assay[30]. On the other hand, GFP emits green fluorescence after being excited by light in the blue to ultraviolet range. Increasing production of GFP increases the intensity of green light emission[30]. Similarly, increasing the production of luciferase increases the bioluminescence intensity[30]. While the researchers of these whole cell biosensors discussed in this review seem to favor β-galactosidase as reporter due to its colorimetric output identifiable by the naked eye, fluorescent protein and luciferase-based reporters need no additional substrates and are more suitable for quantitative measurements over a dynamic range[30].

Both reporters need specialized luminescence reader for quantitative measurements, and while GFP reporters must be excited, luciferase reporters do not. GFP and luciferase also have the advantage of short half-life, that luminescence of the proteins directly correlates with gene expression, for applications in real-time measurements[30]. For preliminary on-site detection of antibiotics in soil and water, the β-galactosidase reporter is more suitable since it can easily be used in a low-tech environment without specialized personnel or equipment. However, for more precise, quantitative determination of antibiotic concentration in samples to provide meaningful data for subsequent antibiotic removal as well as soil and water sample treatment, the luciferase-based reporter is more suitable.

Conclusion

In this review, the suitability of various whole cell biosensors for on-site detection of two different classes of antibiotics, β-lactams and tetracyclines, in farm water and soil was compared on the basis of practicality, performance, range of detection, and ability to accommodate varying pHs and temperatures encountered in the field. Of the β-lactam biosensors, only the *B. subtilis*-based biosensor had both sufficient sensitivity as well as signal stability. However, the *B. subtilis*-based biosensor has a relatively long response time and needs to be modified in order to produce a colorimetric output or to be used with a suitable hand-held luminometer. The *P. aeruginosa*-based biosensor had a shorter response time, but would not be able to detect β-lactams at the levels found in farm water. There is therefore a trade-off between sensitivity and response time for β-lactam biosensors, with sensitivity being the more important consideration. It should be noted that recent methods of antibiotic detection in soil involving mass spectroscopy have a response time of within four hours[31]; thus, the whole cell biosensors discussed in this study all have an advantage in response time over mass spectroscopy methods.

The three tetracycline biosensors had comparable sensitivities, but all had their advantages and disadvantages. The paper strip biosensor is significantly more convenient for on-site monitoring but has a longer response time and requires soil extract. The biosensor by Hansen and Sorenson had a slightly shorter response time than the paper strip biosensor, but had lower sensitivity. The biosensor by Bahl et al. was slightly more sensitive than the paper strip biosensor, but its use of flow cytometry makes it impractical for on-site detection. Few biosensors were tested for pH and temperature sensitivity, as most of the biosensors were only tested in laboratory conditions. Furthermore, only the paper strip biosensor was tested using soil and water samples, and the performance of the other biosensors for these types of samples is unknown. While this review attempts to infer the performance of several biosensors in soil and water samples using pH and temperature ranges for the biosensor bacteria species, the biosensor must be ultimately tested using soil and water samples in the field for accurate analysis. The development of portable equipment for quantitative measurements of the biosensor reporter signal would also improve the practicality of these biosensors. Overall, though the data supports the value of whole cell biosensors as a tool for detection of antibiotics in agricultural samples, more experimentation is needed before the available biosensors can be adequately compared and implemented for on-site detection.

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