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Rapid High-Throughput Assessment of Aerobic Bacteria in Complex Samples by Fluorescence-Based Oxygen Respirometry

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A simple method has been developed for the analysis of aerobic bacteria in complex samples such as broth and food homogenates. It employs commercial phosphorescent oxygen-sensitive probes to monitor oxygen consumption of samples containing bacteria using standard microtiter plates and fluorescence plate readers. As bacteria grow in aqueous medium, at certain points they begin to deplete dissolved oxygen, which is seen as an increase in probe fluorescence above baseline signal. The time required to reach threshold signal is used to either enumerate bacteria based on a predetermined calibration or to assess the effects of various effectors on the growth of test bacteria by comparison with an untreated control. This method allows for the sensitive (down to a single cell), rapid (0.5 to 12 h) enumeration of aerobic bacteria without the need to conduct lengthy (48 to 72 h) and tedious colony counts on agar plates. It also allows for screening a wide range of chemical and environmental samples for their toxicity. These assays have been validated with different bacteria, including *Escherichia coli*, *Micrococcus luteus*, and *Pseudomonas fluorescens*, with the enumeration of total viable counts in broth and industrial food samples (packaged ham, chicken, and mince meat), and comparison with established agar plating and optical-density-at-600-nm assays has been given.

The increased use of microbial tests in food, pharmaceutical, and biomedical industries as well as environmental monitoring and process control has resulted in the demand for rapid, high-throughput, and cost-efficient assays capable of studying bacteria (either specific species or overall populations) in complex samples such as food, broths, waste, and biofermentations. Such assays can indicate the presence of microbial contamination and provide a clearer understanding of the growth characteristics of bacteria, thus allowing optimization of fermentation conditions, determination of toxic and/or metabolic effects of different samples (chemical and environmental) on test organisms, and the study of drug resistance of microbial strains.

Established methodologies include direct methods, such as microscopy and plate counting, and indirect methods, such as turbidometry, immunological assays, and DNA-based techniques, but none of these has proven ideal. Direct microscopic counting has been limited by its inability to distinguish between live and dead cells without treatment with suitable stains, probability of missing relatively small cells, and unsuitability for low cell densities ($<10^6$ cells/ml) (8). Counting of colonies on agar plates, which is routinely conducted in the food, dairy, medical, and pharmaceutical industries (8), provides a reliable estimate of total viable counts, but this method is labor intensive and time-consuming, requiring 1 to 3 days of incubation, and is subjective due to visual assessment. Also, a single colony may be formed by a single cell or by a cluster of cells (5). Detection of specific bacterial strains involves additional steps: nonselective preenrichment, followed by enrichment and plating on selective and differential agars, with subsequent biochemical and serological assays taking up to 7 days (2, 7, 19). Turbidometric measurement of microbial growth (8) is noninvasive, inexpensive, relatively rapid, and amenable to automation but has inherent problems such as the inability to analyze complex (cloudy or colored) samples (10), impracticality for suspensions containing fewer than 10^7 cells/ml or for cells which grow in clumps (20), and nonlinearity at high cell concentrations due to rescattering. For indirect methods such as immunological assays, nucleic acid-based techniques (hybridization probes and PCR) (7, 19), although they provide relatively short assay times of 6 to 18 h and high sensitivity of ~10 CFU/ml (3, 19), identification is still a multistep process requiring specialized reagents, equipment, and trained personnel. Differentiation of viable and nonviable cells by these methods is often problematic, resulting in frequent false positives.

The methodology used in the area of toxicity testing using bacteria varies depending on the application. The Microtox test is a system for rapid (15 min) in vitro assessment of acute toxicity of environmental samples and uses the bioluminescent bacterium *Vibrio fischeri*. However, the use of a special strain of *V. fischeri* limits method flexibility and adequacy and requires controlled salinity and pH (1). This, combined with moderate throughput (approximately 15 samples per h) and problems associated with samples, which are turbid or colored or contain chlorine, limits its use. The Kirby-Bauer method in clinical microbiology is used to determine the sensitivity of a culture to a chemical, while the antibiotic dilution assay is used to determine the MIC of an agent that is required to inhibit growth (9). Inabilities to provide high sample throughput or length of assay time are serious drawbacks associated with these methods. The turbidometric methods described above can also be used to assess general toxicity.

More recently, detection of bacteria via monitoring of consumption and/or release of their key metabolites, such as CO_2 or O_2, has attracted considerable attention. Thus, the optical sensing systems BACTEC (23), BacT/ALERT (22), BD Oxy-

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gen Biosensor (21), and some others (6, 16) have found use in clinical microbiology (detection of *Mycobacterium tuberculosis*), detection of food-borne pathogens, and in drug discovery (cell-based compound screening). These systems usually rely on solid-state sensors or coatings permanently attached to the test vessel (bottom of a glass vial or a microplate well) and specialized samplers and detectors, thus limiting their flexibility and increasing assay costs.

Respirometric screening technology (RST) developed by our team (18) also employs the optical oxygen-sensing approach but in a different format. RST assays operate by means of soluble (dispensable) fluorescence-based oxygen probes (17), and they were adjusted for use on standard microtiter plates (24 to 384 well) and fluorescence plate readers. The oxygen-sensitive probe used in RST is simply added to test samples. The probe is quenched by sample dissolved oxygen in a nonchemical, reversible manner (collisional quenching of fluorescence [17]) and responds to changes in oxygen concentration in a predictable manner by changing its fluorescent characteristics, emission intensity and lifetime, which are being monitored. Molecular oxygen is a key metabolite of aerobic cells and higher organisms, and alterations in cellular oxygen uptake serve as useful markers of their numbers, metabolic status, viability, and responses to various stimuli. The RST assays have been successfully applied to the monitoring of oxygen respiration of the yeast *Saccharomyces pombe* (16), mammalian cells (4), embryos, and whole organisms (13) and to study processes of apoptosis (15) and soil remediation (14).

In this paper we describe further development of a simple, rapid, direct, and robust micromethod based on the RST methodology for the monitoring of growth of rapidly growing aerobic microorganisms, quantification of total viable counts, and responses of bacterial cells to different effectors. General performance evaluation of such respirometric microbial assays and comparison with established techniques, such as turbidimetry (optical density at 600 nm [OD_{600}]) and colony counts on agar plates, are presented followed by validation with complex samples of food homogenates and responses to model toxicants.

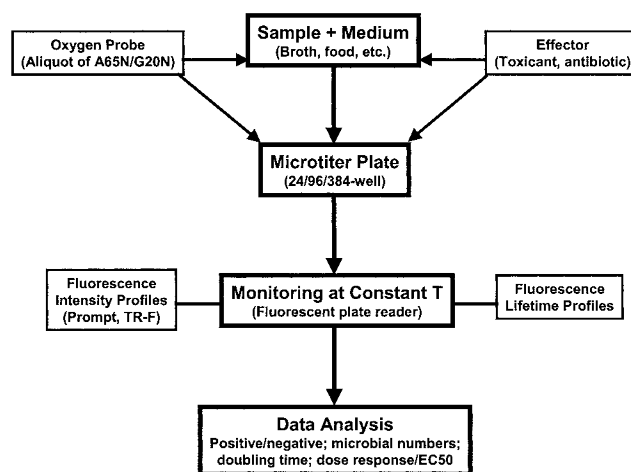

FIG. 1. Flow chart of the respirometric bacterial assay indicating the flexibility of the system with regard to user choice of oxygen probe, microtiter plate, detection mode, and data analysis.

MATERIALS AND METHODS

Materials. Phosphorescent oxygen probes, types A65N-1 and G20N-1, were from Luxcel Biosciences, Cork, Ireland. NaCl, $CuSO_4$, $NiSO_4 \cdot 6H_20$, $Pb(NO_3)_2$, $ZnSO_4 \cdot 7H_2O$, dimethyl sulfoxide (DMSO), yeast extract, tryptone, Luria-Bertani (LB) broth, nutrient broth, standard nutrient agar, penicillin, streptomycin, antimycin A, heavy mineral oil, and Mylar sealing film were all from Sigma-Aldrich, Dublin, Ireland. All the other chemicals and solvents were of analytical grade; solutions were prepared using Millipore water. Standard flat-bottom 96-well microplates made of clear polystyrene were from Sarstedt, Waterford, Ireland.

Culturing of microorganisms. Colonies of microorganism strains were removed from solid agar and used to prepare a suspension in either 100 ml LB broth for *Escherichia coli* (DH5α MCR) or nutrient broth for *P. fluorescens* (wild-type water isolate) and *M. luteus* (ATCC 9341) in a 500-ml flask. Bacteria were then grown at optimal culturing temperature (*E. coli*, 37°C; *M. luteus*, 30°C; *P. fluorescens*, 25°C) and shaken until an OD_{600} of ~0.5 was reached. Cells were enumerated by light microscopy using a standard improved Neubauer hemocytometer (Assistant) and light microscope Alphaphot-2 YS2 (Nikon) as well as on agar plates. These stock solutions were used immediately in subsequent experiments at the required working dilutions in appropriate growth media as described below.

Respirometric analysis of broth samples. According to the manufacturer's instructions, A65N-1 or G20N-1 oxygen probes were reconstituted in 1 ml of water or assay medium to give stock solutions of ~1 μM and ~3 μM, respectively, which were stored in the dark at 4°C for further use. Two-hundred-microliter samples containing bacteria at known concentrations or food sample homogenates were added to the wells of the microplate with the oxygen probe used at a final working concentration of 100 nM (A65N-1) or 300 nM (G20N-1). The plate was then sealed either using Mylar sealing film or with a layer of heavy mineral oil applied on top of each sample (100 μl/well; oil acts as a barrier for ambient oxygen and prevents sample evaporation in long-term experiments) or left unsealed (as shown in Results, sealing was found to have no significant effect on the formation of oxygen gradients in test samples). The microplate was then read on a fluorescence plate reader. For bacterial enumeration, readings in each well were taken every 15 min over an approximately 12-h period. The assay flow chart is shown schematically in Fig. 1. Reliable temperature control during the respirometric assay is necessary, as both oxygen probe response and microbial respiration are temperature dependent. Therefore, microplate readers equipped with active temperature control of the microplate compartment should only be used in these assays.

Fluorescence intensity measurements on a prompt fluorescence reader, SpectraMax Gemini (Molecular Devices), were carried with excitation at 380 nm and emission at 650 nm. Time-resolved fluorescence (TR-F) measurements were carried out on xenon flashlamp-based readers Victor V (Perkin-Elmer Life Sciences, Boston, Mass.) and Genios Pro (Tecan, Mänerdorf, Switzerland) using standard sets of filters of 340 and 642 nm as well as 380 and 650 nm, respectively, with delay and gate times of 50 μs and 70 μs as well as 60 μs and 100 μs, respectively, when using the A65N-1 probe, and 30 μs and 70 μs as well as 30 μs and 100 μs, respectively, when using the G20N-1 probe. TR-F measurements on an ArcDia reader (Luxcel Biosciences, Cork, Ireland) were carried out at 650 nm, using excitation with a solid-state 532-nm laser (square pulses, 20-μs duration), delay time of 10 μs, gate time of 70 μs, and an integration time of 1 s per well. Phosphorescence decay time measurements were also performed on the ArcDia reader under the same conditions, using a built-in multichannel scaler (bin width, 0.5 μs; 200 datum points for each decay curve) giving instantaneous readout of lifetime calculated according to a single exponential fit. Measurement conditions for different instruments are summarized in Table 1.

Measured time profiles of phosphorescence intensity or lifetime were analyzed to determine the time required to reach a threshold level of the probe signal for each sample. For fluorescence intensity measurements, readings for each test sample were normalized for their initial signal at time zero to compensate for possible optical effects of complex samples such as food homogenates, which may alter the probe signal.

Calibration curves for individual strains of bacteria were produced by plotting the time required to reach a threshold intensity versus seeding density of bacteria ranging from 1×10^9 to 1×10^1 cells/ml. Threshold intensity was, in this case, defined as half the maximum signal reached by an average respiration-growth profile as has been used in similar studies by other research groups (21). These calibrations were used to subsequently determine the concentration of bacteria in unknown samples, such as food homogenates. Doubling times of individual

TABLE 1. Measurement parameters for the respirometric assays using oxygen probes A65N-1 and G20N-1 in nutrient broth on a selection of different fluorescent plate readers and corresponding positive, negative, and blank signals (typical values)

Instrument (excitation/emission, in nm)	Detection mode (settings)	Oxygen probe	Signal[b]			Fold increase (positive:negative)
			Blank (no probe)	Negative (21% O_2)	Positive (0% O_2)	
SpectraMax Gemini (Molecular Devices) (380/650)	Prompt fluorescence (continuous excitation)	A65N-1 G20N-1	~250 ~250	~300 ~400	~450 ~2,500	~1.5 ~6
Victor V (PE Life Sciences) (340/642)	TR-F (excitation, 20-μs flash; delay/gate time, 50/70 μs [for A65N-1] and 30/70 μs [for G20N-1])	A65N-1 G20N-1	~40 ~300	~5,500 ~2,500	~60,000 ~1,000,000	~10 ~400
Genios Pro (Tecan) (380/650)	TR-F (excitation, 20-μs flash; delay/gate time, 60/100 μs [for A65N-1] and 30/100 μs [for G20N-1]	A65N-1 G20N-1	~60 ~150	~700 ~350	~5,000 ~40,000	~7.5 ~3.5
ArcDia (Luxcel) (532/650)	TR-F (excitation, 10-μs laser pulse; delay time, 10 μs; gate time, 70 μs)	A65N-1 G20N-1	~250 ~250	~13,000 ~3,500	~45,000 ~400,000	~3.5 ~100
ArcDia (Luxcel) (535/650)	Lifetime based (excitation, 10-μs laser pulse time; scaler settings: bin width, 0.5 μs; no. of data points, 100; fitting, single-exponential decay)	A65N-1 G20N-1	ND[a] ND[a]	~25 μs ~15 μs	~70 μs ~100 μs	~2.5 μs ~6.5 μs

[a] ND, not determined (random values).
[b] Values are in relative fluorescence units unless otherwise specified.

strains at various temperatures were determined from the slopes of calibrations linearized in semilog plots (log base 2 of the bacterial concentration versus the time to reach threshold signal).

Analysis of food samples. According to standard procedures, 10 g from each sample of sliced ham, chicken, or mince meat was blended with 90 ml of sterile peptone water (0.1%) solution for 1 min in a sterile stomacher bag (Stomacher Lab System Model 400; Colworth, London, United Kingdom) using a stomacher (Colworth Stomacher 400; Colworth, London, United Kingdom). Subsequent dilutions were obtained by mixing 1-ml aliquots of homogenate with 9 ml of peptone water (0.1%) solution. These dilutions were then analyzed in parallel by aerobic colony counting on standard nutrient agar plates, and they were enumerated visually after an incubation period of 48 h at 30°C and by the fluorescence-based assay, where they were then dispensed in microwells together with the oxygen probe at standard working dilution and monitored as before on a plate reader to determine times to reach threshold intensities. CFU/ml from the agar plates were then compared to those calculated using calibration curves to convert from threshold times to CFU/ml.

Toxicity testing. To assess the effects of different toxicants [$CuSO_4$, $NiSO_4 \cdot 6H_2O$, $Pb(NO_3)_2$, $ZnSO_4 \cdot 7H_2O$, DMSO, penicillin, streptomycin, and antimycin A], they were added at the indicated concentrations to a constant concentration of test bacteria (~10^8 cells/ml), and respiration profiles produced by the treated as well as untreated bacteria (positive controls) and by samples without bacteria (negative controls) were measured. Measurement setup was similar to that described above, with fluorescent intensity monitored every 2 min for 2 h. In this case, the initial slopes of the probe fluorescence intensity or lifetime increase over time were analyzed and taken as a measure of cellular metabolic response rather than the threshold signals.

Determination of individual bacterial species in mixtures by respirometry. For the determination of individual bacteria species, *E. coli* and *P. fluorescens* cells were cultured individually as described above and then mixed to give 10^7 cells/ml of *P. fluorescens* and 10^4 to 10^1 cells/ml of *E. coli*. These mixed populations were treated with antibiotics (100 mg/liter of streptomycin) and/or subjected to temperature conditions (37°C) that inhibited the growth of *P. fluorescens* but allowed *E. coli* growth, and then the populations were analyzed by respirometry. Time to reach threshold signal was determined as described above and used to assess method selectivity.

Turbidometric assay (OD_{600}). Assay setup was the same as that for the respirometric assay (see above), but no oxygen probe was added to the samples. The microplate was measured on the Tecan Genios Pro plate reader, taking absorbance readings at 620 nm in each well periodically over approximately 12 h. The time to reach an absorbance reading of 0.0825 arbitrary units (approximately three times above the blank signal) was determined and used to plot calibration curves for this assay.

RESULTS

Assay setup for the detection of bacteria in culture via oxygen respiration. The oxygen probes were initially measured on different instrumentation under different conditions which were subsequently used in respirometric experiments with bacteria and food samples. Results summarized in Table 1 show that probe type and instrumentation have pronounced effects on signal values and quality of data generated. Thus, a standard A65N oxygen probe provides satisfactory performance and moderate (1.5- to 7-fold, depending on the measurement mode) signal increase for positive samples. The newly developed G20N-1 probe (12) can be seen to produce greater signal enhancement upon sample deoxygenation and (5- to 100-fold, depending on the measurement mode) thus gives better contrast between positive and negative samples. TR-F detection

provides much higher signals and signal-to-blank ratios, reduces the risk of generating false-negative results (e.g., caused by very weak probe signal from the sample so that signal increase is difficult to detect), and provides better contrast (Table 1, positive:negative signal ratio) than in prompt fluorescence mode. Lifetime-based detection of the probe allows self-referencing and is 100% predictive of the probe signal in such respirometric assays, making it easily distinguishable for both positive and negative samples (e.g., 80 μs and 25 μs, respectively, for the A65N-1 probe; Table 1). At the same time, we have found that signal patterns and final estimates in respirometric experiments were practically the same for all of these different detection modes if the assay was set up and performed properly. Prompt fluorescence readers are more widely available and installed in many microbiological laboratories (for other applications) than TR-F and fluorescence lifetime-based spectrometer readers such as the ArcDia. Therefore, subsequent experiments on microbial respiration were carried out using fluorescence intensity measurements.

Typical respiration profiles of *E. coli* seeded at different concentrations in growth medium containing A65N-1 oxygen probe and monitored at 30°C are shown in Fig. 2a. Sigmoidal curves of the probe fluorescent signal reflect the process of deoxygenation of test samples, which is dependent on the initial number of bacteria and their proliferation rate. Due to cellular respiration, dissolved oxygen in the medium is changing from air saturated at the start of the experiment to almost zero levels in positive samples. At very high seeding cell numbers, initial sample deoxygenation is evident (increased probe signals), while very low numbers require a considerable period of time to reach detectable levels of cellular respiration and sample deoxygenation. One can see that the method provides sensitivity down to a single cell, which for rapidly growing microorganisms such as *E. coli* can be achieved within 10 to 12 h. Negative sample signals remain stable at the baseline level. Typical respiration profiles of *E. coli* obtained in fluorescence lifetime mode are given in Fig. 2b for comparison.

For actively growing and rapidly respiring cells, such as bacteria used in this study, sealing of samples to exclude ambient air oxygen which would otherwise interfere by destroying oxygen gradients associated with cellular respiration appears to be unnecessary and redundant. In this case, a mineral oil seal applied on top of each sample was seen to have a minor effect on signal profiles (Table 2). At the same time, sealing the whole plate with adhesive film (Mylar) was found to be desirable to minimize evaporation and cross-contamination of samples in long-term experiments and to prevent accidental spills.

Monitoring of microbial respiration in broth and enumeration of bacteria. Following the above basic setup and optimization of the respirometric assay, experiments were carried out with *E. coli*, *P. fluorescens*, *M. luteus*, and some other common bacterial strains (data not included) to develop standardized procedures for determination of total viable counts of aerobic bacteria.

All the microorganisms studied were found to produce respiration patterns similar to those of *E. coli* (Fig. 2a and b), while the differences were related to differences in their doubling times. Calibration curves for *E. coli* obtained using the A65N-1 probe for lifetime- and intensity-based measurement, plotted in semilogarithmic scale and presented in Fig. 3, are

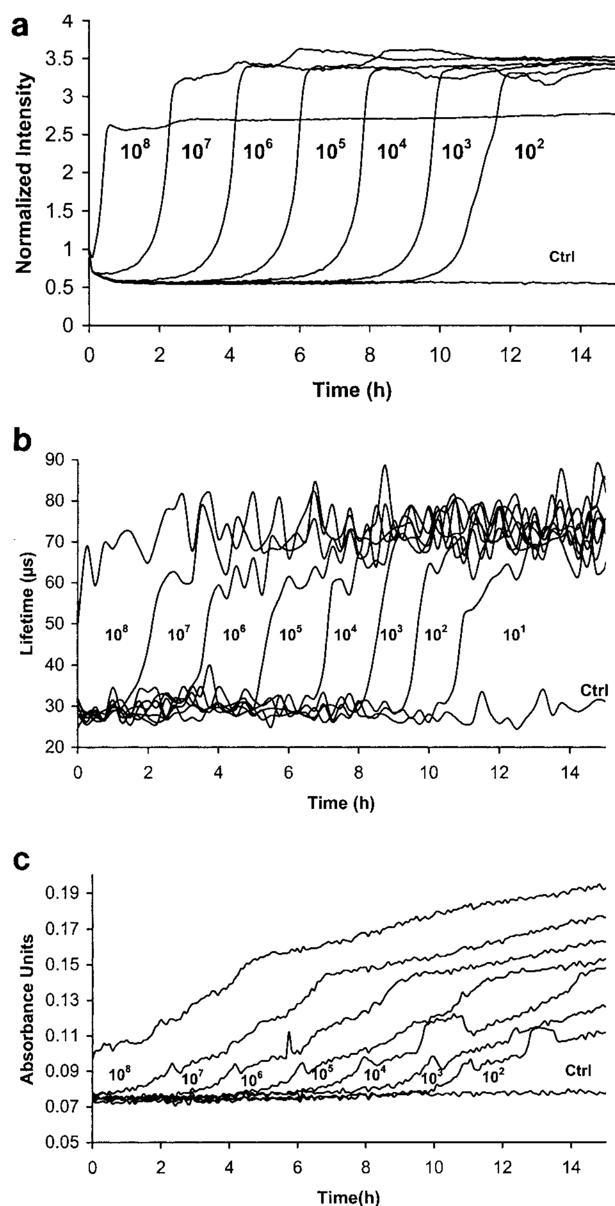

FIG. 2. Growth profiles of *E. coli* seeded at the indicated concentrations in nutrient broth and measured at 30°C (a) by oxygen respirometry in TR-F intensity mode on the Genius Pro reader, (b) by oxygen respirometry in lifetime mode on the ArcDia reader, or (c) by turbidometry on the Genios Pro reader (absorbance at 620 nm).

seen to be linear. Calibration functions obtained with different bacteria and in repeated experiments (on different days) are given in Table 2. These calibrations can be used to determine bacteria in unknown samples based on a recorded time required to reach the threshold signal ($t_{threshold}$). The doubling time of different bacteria can also be determined from the slope of corresponding calibrations or from respiration profiles of several known dilutions of the same stock. At 30°C, doubling times for *P. fluorescens*, *M. luteus*, and *E. coli* were found to be 51 min, 30 min, and 31 min, respectively ($n \geq 4$), which is quite in agreement with the literature. It was also possible to monitor the effect of assay temperature (range, 25 to 40°C) on the

TABLE 2. Calibration equations for the enumeration of *E. coli, P. fluorescens* and *M. luteus* in nutrient broth obtained in different experiments (including repeats)

Bacterium	Temperature, seal	Analytical equation[a]	R^2
E. coli	37°C, oil seal	$t_{threshold} = -0.5235\text{Ln(Conc.)} + 11.807$	0.9939
	37°C, Mylar seal	$t_{threshold} = -0.6049\text{Ln(Conc.)} + 13.516$	0.9935
	37°C, no seal	$t_{threshold} = -0.6592\text{Ln(Conc.)} + 15.107$	0.9949
E. coli	30°C, oil seal	$t_{threshold} = -0.6993\text{Ln(Conc.)} + 14.403$	0.9899
	30°C, oil seal	$t_{threshold} = -0.729\text{Ln(Conc.)} + 14.635$	0.9924
	30°C, oil seal	$t_{threshold} = -0.6321\text{Ln(Conc.)} + 13.983$	0.9979
	30°C, oil seal	$t_{threshold} = -0.7879\text{Ln(Conc.)} + 16.262$	0.9917
M. luteus	30°C, oil seal	$t_{threshold} = -0.7322\text{Ln(Conc.)} + 15.36$	0.9946
	30°C, oil seal	$t_{threshold} = -0.7192\text{Ln(Conc.)} + 15.923$	0.9981
	30°C, oil seal	$t_{threshold} = -0.5949\text{Ln(Conc.)} + 12.729$	0.9959
	30°C, oil seal	$t_{threshold} = -0.8717\text{Ln(Conc.)} + 14.815$	0.9979
P. fluorescens	30°C, oil seal	$t_{threshold} = -1.2874\text{Ln(Conc.)} + 24.028$	0.9966
	30°C, oil seal	$t_{threshold} = -1.2688\text{Ln(Conc.)} + 23.193$	0.9993
	30°C, oil seal	$t_{threshold} = -1.2176\text{Ln(Conc.)} + 22.161$	0.9987
	30°C, oil seal	$t_{threshold} = -1.3029\text{Ln(Conc.)} + 25.57$	0.9912

[a] All concentrations (Conc.) were measured in quadruplicate ($n = 4$).

doubling time of *E. coli* and *P. fluorescens*. Doubling times of *E. coli* over the temperature range decreased from 42.74 (25°C), 31.15 (30°C), 24.84 (35°C), 23.81 (37°C), and 20.41 (40°C) min, while for *P. fluorescens* doubling times decreased from 58.29 (25°C) to 52.68 (30°C); however, no bacterial doubling could be recorded above 30°C due to *P. fluorescens* cell death.

Direct comparison of the new respirometric assays of bacteria in broth with an established turbidometric assay (OD_{600}) is given in Fig. 2c. In this case, growth of the same *E. coli* samples in the same microplate were monitored by simultaneous absorbance readings at 620 nm and fluorescence readings at 380 nm (excitation) and 650 nm (emission) on a Genios Pro reader (Tecan), which has such capabilities. As seen in Fig. 3, the results of turbidometric experiments are almost identical to those of the respirometric assay.

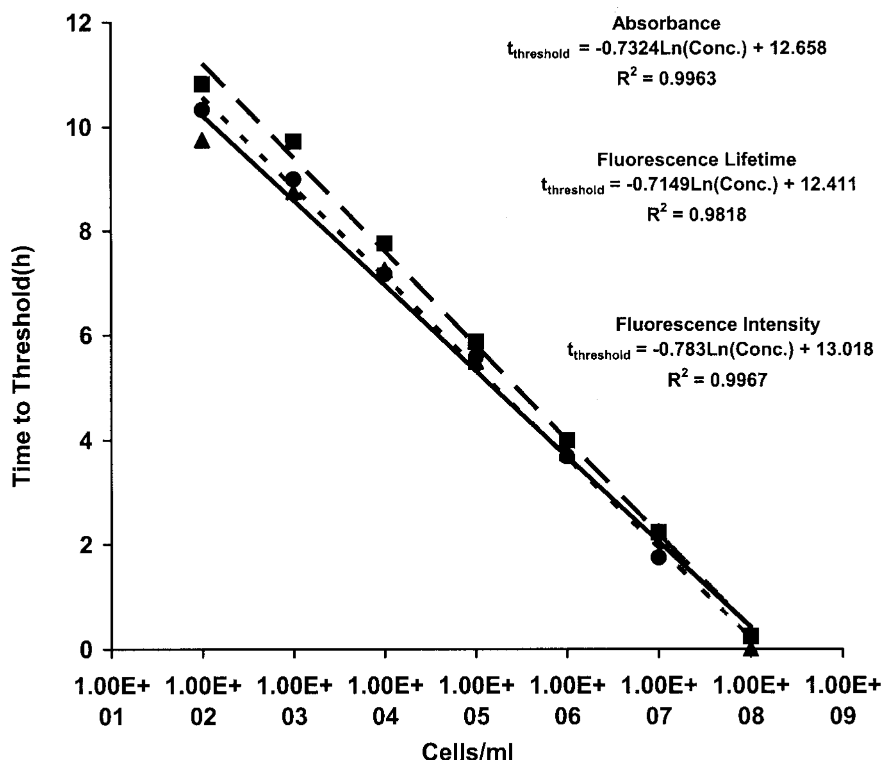

FIG. 3. Calibration curves for quantification of *E. coli* in nutrient broth based on absorbance (●), fluorescence intensity (■), and fluorescence lifetime (▲) measurement modes, as described in the legend to Fig. 2.

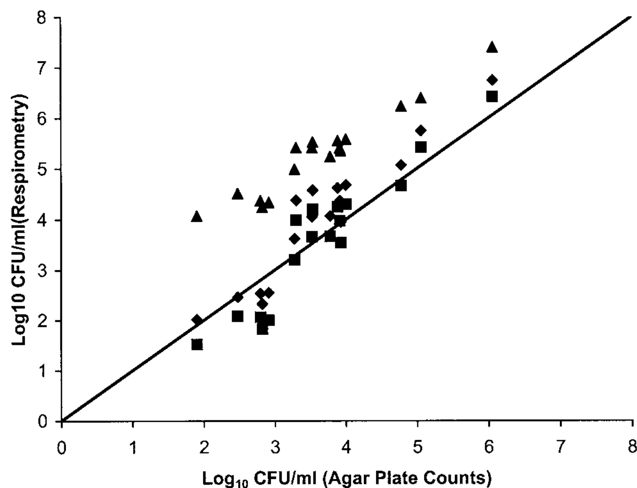

FIG. 4. Correlation of the results of enumeration of aerobic bacteria in food samples by plating on agar (colony counts) and by oxygen respirometry using three sets of calibrations: *E. coli* (■), *M. luteus* (◆), and *P. fluorescens* (▲).

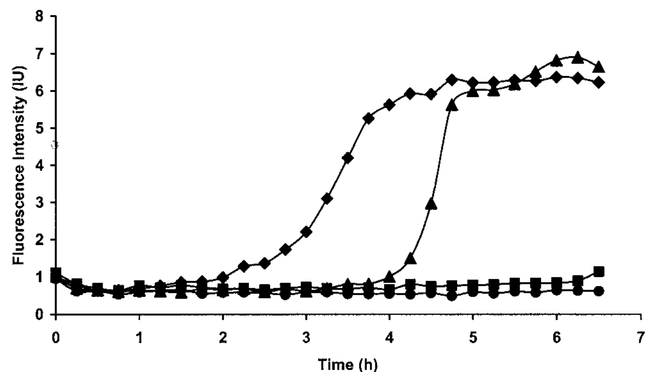

FIG. 5. Determination of *E. coli* in a mixed population of bacteria in nutrient broth based on its specific resistance to certain antibiotics: *P. fluorescens* (◆), *P. fluorescens* with streptomycin (■), *P. fluorescens* and *E. coli* with streptomycin (▲), and control (●). IU, intensity units.

Determination of total viable counts in complex samples (food homogenates). To demonstrate the ability of the respirometric assay to determine CFU in complex samples, it was applied to several different types of food samples (homogenates) ($n = 17$) with unknown microbial counts. All food samples comprising cooked ham, chicken, and mince meat were successfully analyzed by respirometry using a measurement temperature of 30°C, which allows for the growth of a wide range of common aerobic bacteria. To avoid false-negative results (e.g., due to reduced signals from the probe or accidental loss of instrument sensitivity), blanks comprising test samples without the probe were also included in the assay. As expected, blanks were seen to produce considerably lower baseline signals than negatives and positives, thus, discrimination between the last two was reliable. Comparison of results of enumeration of aerobic bacteria in food samples by respirometry (using three sets of calibrations) and by a conventional method on agar plates is given in Fig. 4. One can see good agreement between the two methods. Of the three respirometric calibrations, the *E. coli* one proved to be the least statistically different with regard to the data obtained using the conventional agar plate-based assay. F-test analysis conducted using OriginLab 7.5 software gave F values of 0.07, 0.15, and 7.65 for *E. coli*-, *M. luteus*-, and *P. fluorescens*-based calibration curves, respectively, with respective P values of 0.9903, 0.9566, and 0.0003. The accuracy of the CFU/ml when calculated using the calibration curves either of *E. coli* and *M. luteus* indicates that the food-borne bacteria have growth rates which are similar to those of these bacteria under the specified assay conditions (30°C).

Selective determination of individual bacterial species. By exploiting the different effects that antibiotics and temperature have on individual bacterial species, it was possible to select a single species from a mixed populations of bacteria and enumerate it based on corresponding calibrations. Cultures of *P. fluorescens* and *E. coli* were grown separately and then mixed to give a population which contained 10^6 cells/ml and 10^4 to 10^1 cells/ml of *P. fluorescens* and *E. coli*, respectively. These mixed cultures were then treated to favorably select for the smaller population of *E. coli* in the presence of large excesses of *P. fluorescens*. As can be seen in Fig. 5, treatment with 100 mg/liter of streptomycin kills the population of *P. fluorescens* while at the same time leaves the *E. coli* population unaffected. Incubation of the mixed culture at 37°C also favored the growth of *E. coli* over *P. fluorescens*, as *E. coli* organisms double rapidly, in contrast to *P. fluorescens* at 37°C.

Monitoring responses of bacteria to toxicants. The ability to conduct respirometric and turbidometric microbial assays simultaneously on the same plate reader allowed for their comparison in the analysis of compound toxicity. Dimethyl sulfoxide (DMSO) was used as a model toxicant at concentrations of 0 to 20% (vol/vol), with *E. coli* as the test bacteria at 10^8 cells/ml. Figure 6a shows that both assays produce similar sigmoid-shaped dose-response curves, with calculated 50% effective concentration values (EC_{50}s) of 9.94% (vol/vol) and 6.92% (vol/vol), respectively. The turbidometric assay shows complete absence of cell growth at 10% DMSO and above, suggesting that the cells are dead, whereas the respirometric assay still shows recordable respiration even at 15% (vol/vol) DMSO, indicating that even though these concentrations strongly inhibit bacterial growth, the samples are still metabolically active, i.e., viable. Using relatively high concentrations of test bacteria, such respirometric assays can be performed within an hour.

The ability to monitor bacterial growth and viability via oxygen respiration allowed the rapid screening of the toxicity effects of various compounds on bacteria. The effect of antimycin A, penicillin, streptomycin, and a combination of penicillin and streptomycin on *E. coli* can be seen in Fig. 6b. At the concentrations used (0 to 100 mg/liter), streptomycin had no measurable effect, while antimycin A, penicillin, and penicillin/streptomycin produced EC_{50}s of 6.53, 10.48, and 3.54 mg/liter, respectively. For *P. fluorescens*, penicillin showed no measurable effect; however, antimycin A, streptomycin, and penicillin/streptomycin had EC_{50}s of 103.31, 2.33, and 5.26 mg/liter, respectively.

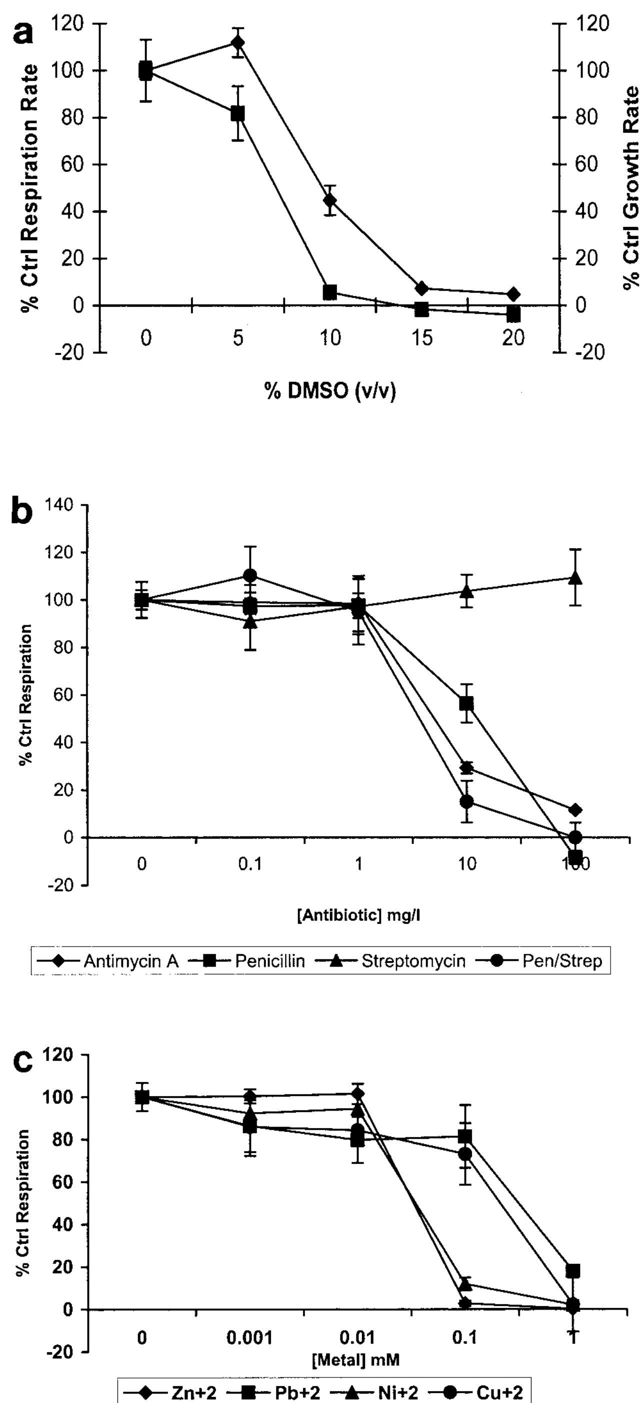

FIG. 6. Dose-response curves of bacteria to different toxicants: (a) *E. coli* to DMSO obtained using the fluorescence-based respirometric (◆) and absorbance-based turbidometric assays (■); (b) *E. coli* to common antibiotics; and (c) *P. fluorescens* to heavy-metal ions, both obtained using the respirometric assay. Measurements were done in nutrient broth medium.

Heavy-metal toxicity is a result of a number of factors, including the fast uptake rate through unspecific transporters resulting in oxidative stress and the inhibition of sensitive enzymes (11). Figure 6c shows the effect of heavy-metal ions on the respiration of *P. fluorescens*. EC_{50}s calculated for Ni^{2+}, Zn^{2+}, Cu^{2+}, and Pb^{2+} were 0.045, 0.061, 0.18, and 0.41 mM, respectively. Using *E. coli* as the test microorganism, no measurable effect was seen for Pb^{2+} (for the concentration range used), while Zn^{2+}, Ni^{2+}, and Cu^{2+} gave EC_{50}s of 0.14, 0.67, and 0.82 mM, respectively. Thus, *P. fluorescens* proved to be the more sensitive of the two bacteria tested, having lower EC_{50}s for all the metals than those of *E. coli*. It should be noted that increasing the sensitivities of these toxicity assays would be possible by allowing longer incubation times of the bacteria in the presence of the toxicant being tested.

DISCUSSION

The new respirometric microassays represent a simple mix-and-measure procedure in which test samples, assay medium, and oxygen probe (and effectors and/or toxicants) are dispensed into the wells of standard microtiter plates (96 or 384 well) and then monitored at a constant temperature on a fluorescence plate reader over a reasonable period of time, usually a few hours. This assay provides a simple, versatile, and convenient alternative to conventional methods for determination of aerobic bacteria in broth and in complex samples such as food homogenates. Such assays are rapid, sensitive, reproducible, and robust, and they allow high sample throughput and can be performed routinely using standard equipment which is already installed in many microbiological laboratories. Real-time monitoring of samples is achieved by direct sensing of dissolved oxygen by fluorescence quenching using a soluble phosphorescent oxygen probe, a commercial reagent produced by Luxcel Biosciences. The probe, which has previously been validated with mammalian cells and small organisms (13, 16), is stable, nontoxic, cell impermeable (4), and used in trace amounts by adding it to growth medium or directly to the samples in microwells. It provides contactless (i.e., noninvasive), real-time monitoring of bacterial oxygen respiration on a microscale in multiple samples in parallel. Furthermore, this methodology also allows selective determination of bacteria in mixtures using group-specific growth conditions (antibiotics and media temperature). Screening of various samples (e.g., chemical, environmental, and industrial waste, natural extracts) for their antibacterial activity, analysis of antibiotic resistance of bacterial strains by sterility testing can also be carried out using this methodology.

Fluorescence intensity signals produced by the oxygen probes at recommended working dilution were sufficiently high for all the instruments. The selection of fluorescent readers used represents the broad spectrum of readers currently in use. Excitation with either UV (340 to 390 nm) or green (530 to 540 nm) light and detection at around 640 to 670 nm can be used. Respirometric data obtained using prompt fluorescence readers (Table 1, Prompt fluorescence), which are the most common today, usually produce satisfactory results with this assay. However, the use of the time-resolved fluorescence (TR-F) or lifetime-based detection mode in a microsecond time domain is advantageous, as this improves the selectivity of oxygen probe detection and reduces the optical interferences, particularly for turbid, colored, fluorescent samples such as food homogenates and environmental and natural extracts, and hence the risk of generating false-negative results.

The ability to enumerate bacteria both in broth and in complex samples with good accuracy (in agreement with established methods), speed (4 to 12 h versus 1 to 3 days for agar colony counts), and sensitivity (down to almost 1 cell per well or 10 cells/ml) and to reliably discriminate between positive and negative samples (especially when using fluorescence lifetime detection) provide high utility for this method and potential for use on a large scale in many areas, particularly in food microbiology, microbial safety, and antibiotic resistance. Comparison of the new method with turbidometry shows that they both cover roughly the same dynamic range and time scale for low cell numbers, but the former provides better contrast and more reliable detection of positive samples. As opposed to turbidometry, it is largely unaffected by sample optical properties, as it relies on measurement of relative changes in probe fluorescence coming on top of the reliably detectable and stable baseline signal (negative control).

Identification of individual microbial species by respirometry exploits the different effects that different media, antibiotics, and temperatures have on the growth rates and doubling times. This approach combines the conventional approaches of providing a favorable growth environment for growth of a single bacterial strain or species with the speed of PCR-based methods in a manner which is nondestructive to the organism being tested and uses small sample size and high sample throughput.

Furthermore, the respirometric assays allowed the monitoring of bacterial responses to toxicants, providing the rapid assessment (1 to 2 h) along with the ability to monitor still viable (respiring) but nongrowing bacteria. It allows for high-throughput screening of potential effectors such as antibiotics, environmental toxins, and compound libraries and is free from problems associated with dark (14), cloudy (10), or autofluorescent samples sometimes experienced with absorbance-based and other methods.

In addition to the relatively standard setup described in this study, microplates of various shapes and from various manufacturers as well as with various commercial instruments can be used to perform such respirometric assays without major modifications (though getting the proper filters for the instrument is required). The assay can be scaled down to 384-well plates without any significant loss of sensitivity and overall performance and with substantial costs savings (i.e., price per assay point). But this and further miniaturization may require automated liquid handling and minor assay optimization. Measurements in 24- and 12-well plates are also possible, though less economical.

By performing the respirometric assays in kinetic mode with periodic measurement of the oxygen probe signal over sufficient periods of time, one can accurately determine the numbers of bacteria in the initial test sample and their doubling rates. At the same time, for semiquantitative or qualitative experiments (the presence of bacteria at above certain threshold concentration, e.g., $>10^4$ cells/ml in food samples), one can simply apply end point measurement following incubation of the test microplate in an incubator at constant temperature (30°C for food samples) for several hours. The exact assay time is determined from calibrations based on the sensitivity required.

As was demonstrated above, selection for this application of a more sensitive G20N-1 oxygen probe over the standard A65N-1 probe, and of the low-cost TR-F and lifetime-based microplate reader ArcDia (Luxcel) over common prompt or time-resolved fluorescence intensity readers, is somewhat advantageous, though not compulsory. Various modifications to the basic assay procedure described in this paper can also be made. Although further validation of the assay is required to allow its use on a large scale, in its present form it looks fairly well developed and acceptable for many research and industrial laboratories performing routine microbiological and bioanalytical tasks.

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