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A sensitive, rapid ferricyanide-mediated toxicity bioassay developed using *Escherichia coli*

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ABSTRACT

A need for rapid toxicity techniques has seen recent research into developing new microbiological assays and characterising their toxicity responses using a range of substances. A microbiological bioassay that determines changes in ferricyanide-mediated respiration for toxicity measurement (FM-TOX) shows particular promise. The development and optimisation of an improved FM-TOX method, incorporating novel features, is described using *Escherichia coli* as the biocatalyst. Omission of an exogenous carbon source, used in previously described FM-TOX assays, substantially improves the assay sensitivity. In addition, the development of a two-step procedure (toxicant exposure followed by determination of microbial respiratory activity) was found to enhance the inhibition of *E. coli* by 3,5-dichlorophenol and four other toxicants, compared to single-step procedures. Other assay parameters, such as the ferricyanide concentration, exposure times and optical density of the biocatalyst were also optimised, sometimes based on practical aspects. Toxicity tests were carried out using the adopted technique on both organic and inorganic toxicants, with classic sigmoid-shaped dose–response curves observed, as well as some non-standard responses. IC_{50} data is presented for a number of common toxicants. The optimised assay provides a good foundation for further toxicity testing using *E. coli*, as well as the potential for expanding the technique to utilise other bacteria with complementary toxicity responses, thereby allowing use of the assay in a range of applications.

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1. Introduction

There has been considerable recent interest in developing rapid micro-organism-based bioassays for determination of toxicity and biochemical oxygen demand (BOD) employing ferricyanide [1–10]. Ferricyanide [$Fe^{III}(CN)_6{}^{3-}$] is a redox mediator that can be used as a terminal electron acceptor in microbial respiration [11]. It is around 50,000 times more water soluble than oxygen, which enables the use of concentrated microbial cell suspensions without ferricyanide becoming limiting to respiration, an important analytical consideration. The resulting production of ferrocyanide [$Fe^{II}(CN)_6{}^{4-}$] as a respiration end product is determined amperometrically from a zero concentration baseline, which enables a rapid and sensitive measure of microbial respiration [8]. The suitability of ferricyanide-mediated respiration (FM-RES) assays for measuring stimulation and inhibition of microbial populations has recently been demonstrated through comparison with standard measures of cell density and viability [12]. This recent study has effectively validated the use of FM-RES assays for toxicity and BOD measurements.

Toxicity assessment using micro-organisms is viewed as desirable for a number of reasons. These include ethical responsibilities, particularly in comparison to toxicity assays using vertebrates, as well as the relative simplicity, speed, low cost and flexibility of the assays [13]. Previously published toxicity assays utilising ferricyanide include the amperometric biosensor Cellsense and the free-cell based MICREDOX assay [14–16]. Cellsense is based on the reduction of ferricyanide to ferrocyanide by immobilised whole cells of a range of species, such as *Escherichia coli*, in a modified electrode [14]. Cellsense is capable of monitoring inhibition of activity in real time, but was shown to be less reproducible than two methods employing cell suspensions by dos Santos et al. [17].

MICREDOX uses a suspension of bacteria cells exposed to a toxicant in the presence of ferricyanide for a defined time period [18]. Inhibition of activity by the toxicant is determined by comparison to a control sample. To date, MICREDOX has been demonstrated

Abbreviations: BOD, biochemical oxygen demand; FM-BOD, ferricyanide-mediated biochemical oxygen demand; FM-RES, ferricyanide-mediated respiration; FM-TOX, ferricyanide-mediated toxicity; GGA, glucose glutamic acid; IC, inhibitory concentration; IQ, inhibition quotient; OECD, organisation for economic co-operation and development; PB, phosphate buffer; rcf, relative centrifugal force; UNSW, University of New South Wales.

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with *E. coli*, *Bacillus subtilis* and *Pseudomonas putida* with 3,5-dichlorophenol (3,5-DCP) as a model toxicant [15]. Pasco et al. [18] also used MICREDOX to study differences in the inhibitory properties of various chlorinated phenol compounds, with *E. coli* and *Klebsiella oxytoca* as biocatalysts, though optimisation of the method focused on reducing the duration of the assay rather than improving sensitivity or reproducibility [18]. Most recently, Liu et al. [19] applied a similar non-optimised *E. coli* assay to a number of different classes of toxicants, including elements such as arsenic and several heavy metals. Minimal toxic responses were observed for Pb^{2+}, Ni^{2+} and Cu^{2+} at concentrations up to 160 mg L^{-1} in the study, despite Cellsense, using *E. coli* as a biocatalyst, reporting an IC_{50} value of 6.5 mg L^{-1} for Cu^{2+} [20]. Therefore, there are sensitivity issues in the most recent version of the FM-TOX assay which need to be addressed through systematic optimisation of the assay parameters.

Each of the previously described ferricyanide-mediated toxicity (FM-TOX) bioassays include, as part of the procedure, addition of the standard BOD_5 glucose glutamic acid (GGA) mixture as a substrate for cellular metabolism. However, Catterall et al. [12] recently demonstrated that addition of GGA ameliorated the toxicity of 3,5-DCP dramatically, thereby reducing the sensitivity of the assay, even at a GGA concentration of only 25 mg L^{-1}. The effect of added GGA solution on Ag^+ toxicity was much smaller over the same range of GGA concentrations; only being significant when the highest GGA concentration (400 mg L^{-1}) was exposed to the lowest Ag^+ concentration (2.5 mg L^{-1}). The inhibition quotient calculations in both Pasco et al. [18] and Liu et al. [19] have reported the 'inhibition of respiratory stimulation by GGA', rather than a straightforward inhibition of total respiration. This can be demonstrated by investigating Eq. (1) [18] and Eq. (2) [19], respectively.

$$IQ_p = 100 \times \left[1 - \left(\frac{S_g - C_e}{C_g - C_e}\right)\right] \quad (1)$$

$$IQ_l = 100 \times \left[1 - \left(\frac{S_g - S_e}{C_g - C_e}\right)\right] \quad (2)$$

where C_e is the endogenous control (no toxicant, no GGA), C_g is the GGA control (no toxicant, with GGA), S_e is the endogenous sample (with toxicant, no GGA) and S_g is the GGA sample (with toxicant and GGA).

These equations both aim to measure the inhibition of stimulation of respiration caused by the added GGA, but approach the calculation of respiratory inhibition in different ways. Eq. (1) does not discriminate between endogenous and stimulated respiration in the toxicant sample (S_g), and can therefore obtain IQ_p values of greater than 100%. This casts doubt on the validity of reporting an IC_{50} value, which should represent a point at which 50% inhibition of the maximum respiration occurs and therefore a result greater than 100% inhibition should not be possible. Eq. (2) improves on this somewhat, using an endogenous correction for both the sample and control, enabling reporting of IQ_l values that can be clearly defined as 'inhibition of respiratory stimulation' between 0 and 100%. However, this approach necessitates the use of two separate samples for each data point obtained, doubling the quantity of reagents used and analysis time. Catterall et al. [12] reported decreases of net respiration of more than 80% compared to endogenous controls in test samples containing Ag^+ and no GGA, validating the potential of an FM-TOX approach without the need for addition of GGA. More importantly, the presence of GGA was found to decrease the inhibition of respiration due to 3,5-DCP almost proportionally. Therefore, the exclusion of exogenous substrate was considered here for the further development of the FM-TOX approach.

In addition, existing FM-TOX assays utilise a single-step protocol, in which the toxic exposure and measurement of respiration are combined. Michaelis–Menten uptake kinetics dictate the ability of a toxicant to exert its effect on cells within a solution, so the full toxic effect of a compound is unlikely to be observed at the start of a single-step toxicity assay. Supporting this, empirical data indicates that the toxic effect of heavy metals on *E. coli* in the Cellsense test takes around 20 min to stabilise [20], with a similar lag time for the effect of 3,5-DCP on activated sewage sludge [16]. This helps to explain the results described in Pasco et al. [18], in which short, single-step FM-TOX assays were much less sensitive than longer assays. It is, therefore, justifiable to investigate the potential use of a preliminary toxicant exposure step in the FM-TOX procedure, in which the toxicant in a test sample is allowed to exert its effect before ferricyanide is added, to commence the quantification of microbial respiration.

An ideal toxicity assay should be rapid, inexpensive, simple, consistent and sensitive over the desired concentration range for a given application. Development of the MICREDOX toxicity assay focused on the first three of these parameters [18], but further development is necessary to ensure the results obtained are meaningful and provide sensitive, reproducible results. The objective of this study was to develop and optimise an FM-TOX assay using *E. coli*. The results of key optimisation experiments are described, and the adopted conditions for the FM-TOX assay are summarised. Toxicity responses and data for a range of substances are presented for which ideal dose-response curves were obtained. We also discuss some observed limitations of the assay.

2. Methods

2.1. General methods

E. coli K12 was obtained from the UNSW Culture Collection. Cultures were maintained on nutrient agar plates (Oxoid) and replated regularly to ensure viability. A 100 mL solution of autoclaved tryptone soya broth (TSB, Gibco) was inoculated with a colony of *E. coli* and grown aerobically at 37 °C for 16–18 h on an orbital shaker. A 1 L secondary culture was prepared by inoculating TSB with a 10% (v/v) aliquot from the initial culture. The secondary culture was grown at 37 °C until late exponential phase (approx. 4 h) and harvested by centrifugation at 2800 rcf. Cells were washed twice in phosphate buffer (PB; 0.08 M KH_2PO_4, 0.12 M K_2HPO_4, pH ~6.8), resuspended in PB and adjusted to the desired optical density at 600 nm (OD_{600}).

All reagents used were analytical grade and all solutions were prepared using deionised (MilliPore Element) water. Ferricyanide was prepared in PB, while all toxicants (3,5-DCP, 2,4-DCP, Ag^+, Hg^{2+}, Se(IV), Cu^{2+}, Ni^{2+}, Zn^{2+}, Pb^{2+}, sodium dodecylsulfate (SDS) and I_2) were freshly prepared in Milli-Q water from high-concentration stock solutions. Stock solutions were prepared freshly or stored with appropriate preservation measures, such as acidification to pH 2 for metallic ions.

2.2. General assay and method development

Basic FM-TOX assays utilise several simple key protocols. Microbial cells at a defined optical density are exposed to toxicants in solution. Ferricyanide added to the incubation mixture is used by the cells as a terminal respiratory electron acceptor, and the reduced product, ferrocyanide, is quantified using chronoamperometry. The respiratory activity of toxicant samples relative to control samples was then used to determine respiratory inhibition due to the toxicant.

A number of experiments were carried out to identify optimal or suitable concentrations of ferricyanide and optical density. In addition, the introduction of an initial toxicant exposure period,

in which the microbial cell suspension is exposed to the toxicant without the presence of ferricyanide, was investigated in detail. The optimal duration of the first and second steps in this novel exposure protocol was tested with toxicants or without toxicants (the latter to characterise the endogenous response). The model toxicant 3,5-DCP was used in most validation assays, though 2,4-DCP, Ag^+, Ni^{2+} and Se(IV) were also utilised to make sure that a range of toxicant behaviours were investigated.

At the completion of each assay, the biochemical reaction was terminated by centrifugation at 13,660 rcf, after which the supernatant was decanted into a fresh sample container and allowed to cool to room temperature. Determination of microbially produced ferrocyanide in the supernatant was carried out by chronoamperometry using a 25 μm Pt microelectrode, as described in Morris et al. [4]. Limit of detection (2.3 μM ferrocyanide) and limit of quantification (7.7 μM ferrocyanide) data were calculated from six blank samples (phosphate buffer solution).

Percentage inhibition was calculated using the following equation

$$\%Inhibition = 100 - \left(100 \times \frac{S}{C}\right) \quad (3)$$

where S is the response of the test substance and C is the control response (as limiting current, nA).

Prior to testing, interactions between the toxicants and ferrocyanide were investigated using cyclic voltammograms (CVs), as described in Liu et al. [19]. Toxicants at a concentration of 500 mg L^{-1}, or the limit of solubility if it was lower than this concentration, were added to a solution of 55 mM ferricyanide and 5 mM ferrocyanide and the CVs were compared before and after the addition.

2.3. Adopted toxicity test

The final assay protocol was based on two consecutive steps. First, 1.00 mL *E. coli* suspension (final OD 4.0) was exposed to 1.00 mL test substance (toxicant) for 60 min. Second, 200 μL of 660 mM ferricyanide was added (final concentration 60 mM) and the mixture was incubated for a further 15 min. Test substances comprised toxicants of varying concentrations in Milli-Q water, as well as endogenous control samples which contained only water in the test sample. Unlike previously reported assays, GGA was not included in any samples. The analytical determination and inhibition calculations were conducted as described in the assay development Section 2.2.

For each toxicant, a range of toxicant concentrations were tested. IC_{50} values were determined if the inhibition results met the following criteria:

- At least six samples must be within the 10–90% inhibition range, with at least one sample below 30% inhibition and at least one sample above 70% inhibition.
- The linear regression used for calculation of the 50% inhibition value must have an R^2 value above 0.95.

For the purposes of assay validation, these criteria were viewed acceptable, but increased confidence could be gained by enforcing more stringent criteria, such as the use of duplicate samples at each concentration, a larger number of data points within the useful range and replication of each toxicity experiment to obtain mean IC_{50} results. Log transformation was used where necessary to improve the linearity of the data and hence improve confidence in the calculated IC_{50} value. Individual 95% confidence intervals were determined using SPSS 17.0 (SPSS Inc. 2008).

3. Results and discussion

3.1. Method development

Preliminary optimisation focused on a technique similar to that described by Pasco et al. [18], utilising a single incubation step with GGA included as a substrate. It was assumed that increasing the analytical signal – in other words, the amount of microbially produced ferrocyanide – would improve the sensitivity of the assay. A GGA concentration of 1500 mg L^{-1} was found to produce the maximum endogenous response in these early optimisation experiments. However, toxicity curves for 3,5-DCP were consistently poorer than expected and did not produce quantitative IC_{50} values. Furthermore, stimulation responses (effectively, negative inhibition) were often observed, which is not ideal in toxicity assays using synthetic solutions.

Results from Catterall et al. [12] clearly demonstrate that the inclusion of GGA in FM-RES assays dramatically decreases toxicity of 3,5-DCP to *E. coli*. This effect was observed even with GGA concentrations as low as 25 mg L^{-1} BOD_5 and increased steadily with increased GGA concentration (up to 400 mg L^{-1}). This was such an important observation that we decided that it should be published in advance of the rest of this development study, as part of the more general study on the stimulation and inhibition of *E. coli* respiration, as measured by the FM-RES bioassay [12]. As a result of those previous studies, which have been confirmed by subsequent experiments (data not shown), the optimal concentration of GGA was established to be 0 mg L^{-1}. All optimisation experiments involving the ferricyanide concentration (data not shown) also confirmed the results of previous studies [3,4]; that 55–60 mM ferricyanide is the optimal concentration for FM-RES assays.

The effect of GGA may also explain the results reported by Pasco et al. [18], in which the inhibitory effect of 3,5-DCP on *E. coli* respiration decreased with decreasing single-step assay time, as the ameliorating effect of GGA decreased as it is consumed. Essentially, the toxic effect of 3,5-DCP on *E. coli* becomes more pronounced over time. As mentioned in the introduction, this temporal effect has also been directly observed using 3,5-DCP with activated sewage sludge [16] and with Hg^{2+}, Cu^{2+}, Zn^{2+} and Ni^{2+} in a Cellsense sensor using *E. coli* [20].

A separate exposure step, in which the toxicant could exert its inhibitory effect prior to the addition of ferricyanide, was included to counteract these observations. The decision to employ a two-step assay protocol is a fundamental change in the approach to FM-TOX. The previous single-step approach combined toxicant exposure with measurement in one time period. However, a two-step assay allows a defined toxicant exposure period, in which any toxic effect can develop and stabilise, followed by a short, ferricyanide-mediated incubation to quantify the microbial activity as modified by the toxicant. The two-step technique, therefore, utilises the same general 'exposure-measurement' approach as many standard toxicity tests, including Daphnia [21], respiration inhibition [22] and MicroTox® [23].

To ensure that the response of a two-step assay would be predictable and not affected by the optical density selected, the linearity of the endogenous (no toxicant) response across a range of optical densities was examined. Fig. 1 shows the endogenous response of samples between OD 1 and 8 with a 15 min ferricyanide incubation and 0, 30 and 60 min preliminary exposure periods. A 15 min ferricyanide incubation was selected as previous work has shown this to be a sufficient duration for accurate measurement of microbial respiratory activity [12]. Any shorter measurement time will produce too little ferrocyanide relative to the limit of quantification (7.7 μM ferrocyanide) with little practical benefit in reduced assay time. Longer exposures to ferricyanide may also cause toxic

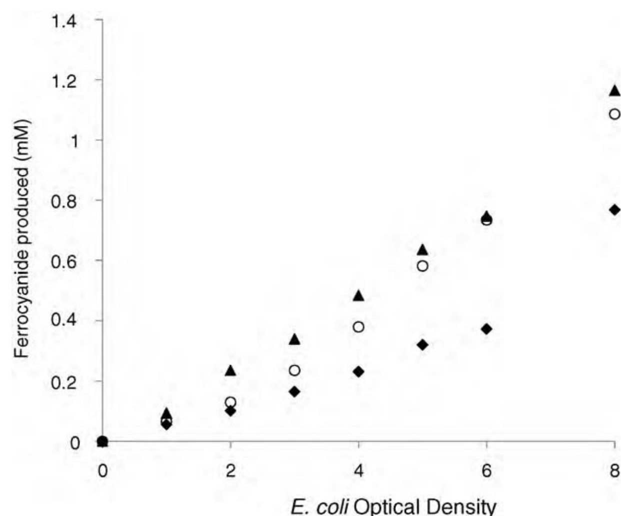

Fig. 1. Endogenous activity of *E. coli* at a range of optical densities (OD) with a 15-min measurement after no preliminary exposure step (closed diamonds), 30 min pre-exposure (open circles) and 60 min pre-exposure (closed triangles).

effects [24], which is another important benefit of a two-step assay that limits the time of exposure to ferricyanide.

The endogenous activity with a 60 min pre-exposure was higher than for 0 and 30 min pre-exposures of the same duration, which is desirable as a larger analytical signal enables more sensitive determination of samples at high inhibition (low ferrocyanide concentration) values. The 60 min pre-exposure had a linear R^2 value of 0.985 across OD 1–8, while the 30 min pre-exposure had an R^2 value of 0.968 across the same range, indicating a more consistent relationship between OD and ferrocyanide production for the 60 min pre-exposure. In the data shown in Fig. 1, and in the results of other experiments, the linearity of the response was sometimes observed to decrease above an OD of 4 or 6.

Development of other parameters were carried out with toxicity experiments using a concentration gradient of the model toxicant 3,5-DCP, with supporting information gained by the replication of tests using other toxicants. As a result, the exact value of each parameter may not be fully optimised for sensitivity to a particular toxicant, but represents a reasonable combination of characteristics. For some parameters, the experimental conditions could be varied without the assay being affected dramatically.

The duration of the toxicant exposure step was investigated further as part of concentration-response experiments, with 90 and 180 min toxicant exposure periods generally found to be unsuitable due to decreases in both the endogenous signal and the toxic response. These assay times are also becoming impractical for a quick assay. Concentration–response curves obtained with 30 and 60 min exposure times with a range of ODs showed generally similar responses for Ag^+ and Zn^{2+}, with a slightly more sensitive response observed at 60 min for 3,5-DCP (data not shown). Therefore, the initial exposure time could be varied between 30 and 60 min depending on the importance of rapid analysis. A 60 min exposure time was used in the remainder of this study, as the endogenous activity was highest at the relevant optical density (see above), and to ensure sufficient time for the stabilisation of any toxic effect [18].

Fig. 2 shows the relationship between optical density and percent inhibition of respiration caused by a range of 3,5-DCP and Zn^{2+} concentrations. The highest sensitivity occurred at the lowest optical density, which is to be expected as the amount of available toxicant per cell is greatest at low cell densities. However, at low optical densities, the quantity of ferrocyanide produced in the 15 min measurement step is relatively low. An OD of 4 was

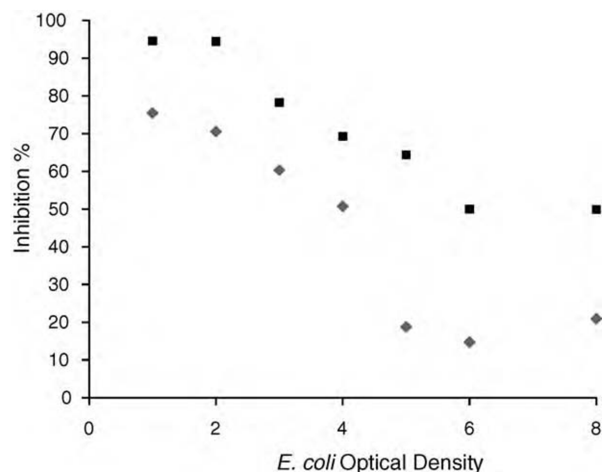

Fig. 2. Relationship between optical density and toxic response for 5 mg L^{-1} 3,5-DCP (open diamonds) and 9.1 mg L^{-1} Zn^{2+} (closed squares).

generally considered most suitable as a compromise between the opposing trends of dilution of the toxicant amongst the microbial population with increasing OD and the amount of ferrocyanide produced by the endogenous control (about 500 μM at OD 4, from Fig. 1) which decreases with OD. However, other OD values still allow for toxicity measurements to be made and may be appropriate in some circumstances. At OD 4 the effective working range of the electrochemical signal, in terms of ferrocyanide concentration produced and measurable, was from 7.7 to 500 μM; nearly two orders of magnitude and therefore provides sufficient sensitivity for a toxicant assay that requires measurement over one order of magnitude (effectively from 10 to 90% inhibition).

Table 1 shows the range of assay characteristics tested and the adopted value or suitable ranges to allow further selectivity for each characteristic (with the preferred conditions in bold). For the rest of this study, a 60 min toxicant exposure and optical density of 4 were utilised.

Following the optimisation experiments, this two-step assay was compared with existing FM-TOX assay protocols. Four assay configurations were tested, summarised in Table 2, against five toxicants (3,5-DCP at 10 mg L^{-1}, 2,4-DCP at 40 mg L^{-1}, Ag^+ at 4 mg L^{-1}, Ni^{2+} at 5 mg L^{-1} and Se(IV) at 4 mg L^{-1}). Toxicant concentrations were selected based on information gathered in the literature and during the optimisation process to ensure they were within a measurable inhibition range. Three of the four assay configurations (B, C and D) are based on assays which have been employed in

Table 1
Summary of FM-TOX assay characteristics tested during development. Values in bold were used for all following studies.

Characteristic	Values tested	Adopted value
Initial exposure (no ferricyanide)	0, 30, 60, 90 and 180 min	30–**60** min
Measurement step (with ferricyanide)	15, 30 and 60 min	**15** min
GGA added	0, 25, 100, 400 and 1500 mg L^{-1} BOD_5 [12]	[a]**0** mg L^{-1} BOD_5
Ferricyanide concentration	5–100 mM	[a]55–**60** mM
Micro-organism density	OD_{600} 1–8	2–**4.0**

[a] These conditions should be considered to be optimal.

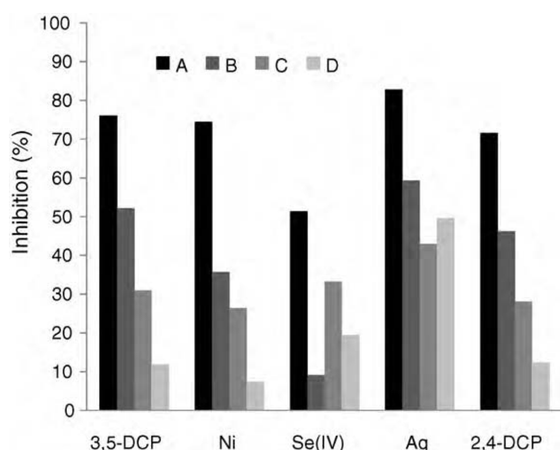

Fig. 3. Percentage inhibition of the respiratory activity of *E. coli* by 5 toxicants under different assay configurations, as described in Table 2. Data are the mean of duplicate samples.

ferrocyanide-mediated assessment of toxicity previously, specifically, Catterall et al. [12], Liu et al. [19] and Pasco et al. [18]. Protocol A is the two-step assay developed in this study with the experimental conditions summarised in Table 1. Percentage inhibition was calculated using Eq. (3) for each set of exposure conditions, with the control samples for each measurement not containing the toxicants.

There are several key results in Fig. 3. There is a clear difference between assay protocols for most toxicants, with protocol A yielding the greatest percentage inhibition for all toxicants. It is consistently more sensitive than protocol B, which is identical with the exception of the 60 min initial toxicant exposure step. As this effect was uniform across a range of toxicant classes, it indicates that there is a time delay between initial toxicant exposure and full inhibitory effect in toxicity assays.

The second major variable was the addition of GGA in protocols C and D. Previous work has shown the addition of GGA to mitigate the toxic effect of 3,5-DCP and, to a lesser extent, Ag^+ in ferricyanide-mediated assays [12]. This effect is also shown in Fig. 3. Protocols C and D, which contained GGA, were less sensitive than protocols A and B, which did not contain GGA, in all tests except for Se(IV), where C and D were more sensitive than protocol B. The 5 min form of the test, protocol D, was less sensitive than the 60 min protocol, C, in four out of five tests. This is expected, as previous studies, as discussed in the introduction, support the general idea that toxic effects may take time to stabilise after initial exposure [16]. Therefore, the results in Fig. 3 indicate that the development process described here has led to an FM-TOX assay protocol which is more sensitive than any previously published assay, predominantly due to the introduction of an exposure step and the omission of GGA.

Toxicant screening for interferences identified several substances where an interfering process was observed. Four toxicologically relevant transition metals, Cu^{2+}, Cd^{2+}, Ni^{2+} and Zn^{2+} were found to precipitate with ferrocyanide and therefore decrease the analytical signal. In combination with the effect of GGA on toxicity measurements, these processes may explain the substantial underestimation of metal toxicity reported previously [19]. The

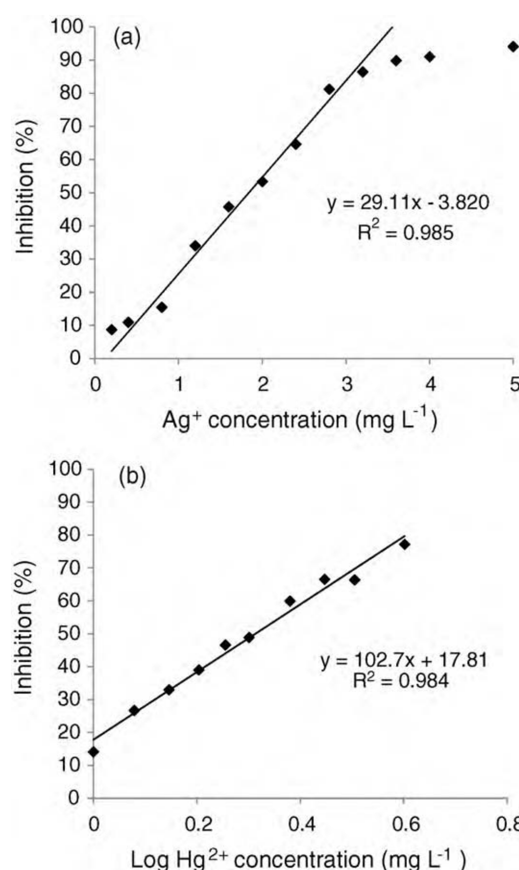

Fig. 4. Concentration–response curves for *E. coli* with (a) Ag^+ and (b) Hg^{2+} using the optimised FM-TOX assay. The linear trendline used for IC_{50} determination is apparent.

effect of these metals on FM-TOX techniques has been fully characterised and is described in a following study (Robertson et al., in preparation). This characterisation process has also demonstrated that, for the purposes of obtaining IC_{50} values using the optimised FM-TOX assay (see below), this effect was very minor (<10%).

3.2. Obtaining toxicity data with the optimised toxicity test

The optimised FM-TOX assay produced classic sigmoidal dose–response curves for a number of test compounds. Fig. 4a and b shows the response of *E. coli* to Ag^+ and Hg^{2+}, respectively. To determine an IC_{50} value based on the data, the raw concentration values were converted to inhibition percentages using Eq. (1). Next, the IC_{50} was determined by solving a linear regression equation. For some toxicants, a log transformation was used to improve the linearity of the data, and meet the curve fitting requirements stated in Section 2.3, such as for Hg^{2+} in Fig. 4b.

Toxic responses from a range of different classes of chemicals, including chlorinated phenols, heavy metals and inorganic toxicants, were determined (Table 3). The IC_{50} values varied over an order of magnitude, with Se(IV) the most toxic and 2,4-DCP the least toxic of those quantitatively analysed. We expected the Hg^{2+} result to be lower and it is possible that this result may reflect

Table 2
Summary of parameters used in the toxicity comparison test.

Protocol	OD	Initial exposure time	Ferricyanide incubation time	GGA added	Based on reference
A	4.0	60 min	15 min	None	Two-step, this study
B	4.0	Not used	15 min	None	[12]
C	6.0	Not used	60 min	198 mg BOD L^{-1}	[19]
D	6.0	Not used	5 min	198 mg BOD L^{-1}	[18]

Table 3
IC_{50} values and individual 95% confidence intervals for the toxicants tested.

Toxicant	IC_{50} (mg L^{-1})	95% confidence interval	Number of points	R^2
3,5-DCP	4.88	3.4–7.8	7	0.983
2,4-DCP	28.2	21.4–38.9	6	0.983
Ag^+	1.84	1.55–2.25	7	0.985
Hg^{2+}	2.03	1.78–2.31	9	0.987
Cu^{2+}	3.71	2.9–5.0	8	0.985
Zn^{2+}	7.5	6.2–9.3	11	0.994
Cd^{2+}	7.8	4.8–15	9	0.975
I_2	13.0	11.2–14.9	9	0.979
Ni^{2+}	*1.9**		*5*	*0.975*
Pb^{2+}	*20.4***		*6*	*0.948*
Se(IV)	*1.41***		*21*	*0.921*

Note: Italicised data did not meet the criteria for linear regression (see text for discussion).
* Less than 6 points used for regression.
** R^2 value < 0.95.

a stability issue with the mercury solution used, although it was made freshly and preserved in acid (pH < 2). The commonly used reference toxicant 3,5-DCP gave an IC_{50} of 4.88 mg L^{-1}, which is similar to previously reported MicroTox® and FM-TOX values of 3.39 mg L^{-1} [25] and 8.0 mg L^{-1} [19], respectively. Interestingly, the results of our optimised FM-TOX assay for Ni^{2+} and Cu^{2+} indicate relatively high toxicity (1.9 and 3.71 mg L^{-1}, respectively), but Liu et al. [19] reported no toxic effect of these metals. This is likely to be due to the more sensitive assay conditions; as shown in Fig. 3, the toxicity of 5 mg L^{-1} Ni^{2+} in the 1-h assay with GGA was very much lower than in the two-step technique presented in this paper (26% vs. 76% inhibition, respectively).

Importantly, the values in Table 3 demonstrate that toxicity determination based on inhibition of the endogenous respiration of *E. coli* is both feasible and sensitive, contrary to findings reported in previous versions of FM-TOX assays [19,26]. In those studies, GGA was added to each test sample, and the suppression of the respiratory stimulation by GGA by the toxicant was quantified. This necessitated quantification of IC_{50} values based on a fraction of metabolic activity, rather than net metabolic activity. However, as shown in Catterall et al. [12], the ability of toxicants to suppress the stimulatory effect of GGA is not consistent, with 3,5-DCP and Ag^+ affecting *E. coli* in different ways as the GGA concentration varied. Therefore, as the calculation used in the present paper determines inhibition of the total metabolism of the cell suspension, the potential confounding effect of added substrate on inter-toxicant comparability of IC_{50} values is eliminated. Collection of more detailed data across the inhibition range for each toxicant tested in Table 3 would enable quantitative calculation of IC_{20} and IC_{80} values in addition to the reported IC_{50} data.

Replicate toxicity tests, on different *E. coli* cultures, were performed for several toxicants. Ag^+ gave a mean IC_{50} value of 1.94 mg L^{-1} with an RSD% of 9.68% (n = 4). Reproducibility between tests with 3,5-DCP was less reliable. There was generally good agreement between tests at low concentrations (up to 50–60% inhibition), but above this point, a plateau in toxic response around 60–70% inhibition, at 3,5-DCP concentrations of up to 50 mg L^{-1} or more, was observed. Inhibition above 90% was not observed for any sample containing 3,5-DCP. This may be due to the mode of action of 3,5-DCP, which can include respiratory uncoupling or direct interference with respiratory transport chain proteins [27]. It is possible that a background level of ferricyanide reduction can take place even if parts of the respiratory system are compromised. For example, a small proportion (14–16%) of ferricyanide reduction in exponentially growing *E. coli* remains even in the presence of high concentrations of CN^-, a strong, well-characterised respiratory inhibitor [28], and a similar effect may occur with 3,5-DCP. This may not be evident in the assays described by Pasco et al. [18] and Liu et al. [19], as their approaches measure an inhibition of only a proportion of the total respiration and do not quantify changes in endogenous metabolism.

During the testing of substances, there were two toxicants which did not provide quantitative test results. Inhibition curves for selenium (IV) and Pb^{2+} failed the requirement for a linear regression R^2 value above 0.95 for data points in the 10–90% inhibition range (Fig. 5a and b). Semi-quantitative IC_{50} values were estimated from the best linear fit which could be obtained with the data available (Table 3). Additionally, an IC_{20} for Pb^{2+} was calculated using 6 points between 0 and 50% inhibition, with a value of 4.65 mg L^{-1} and an R^2 of 0.99.

The response of *E. coli* to these substances is likely to be due to physiological characteristics of the bacteria. The unusual two-stage toxicity curve for Se(IV) is difficult to explain, as the mechanism for Se(IV) toxicity is not well characterised [29]. *E. coli* can reduce selenite and selenate to elemental selenium [30], potentially mitigating some toxic effect, or there may be a rate-limiting step in the uptake of selenium which leads to the variation around the 50% inhibition area in Fig. 5a.

Pb^{2+} is known to be bound by the membrane of *E. coli* to a greater extent than Cu^{2+}, Ni^{2+} and a number of other trace metals [31], and previous toxicity studies have reported substantially lower toxicity for lead than copper or silver [32]. This membrane sequestration may limit the maximum toxic effect within the concentration range tested, leading to the plateau at 65–75% inhibition observed in the concentration range between 50 and 400 mg L^{-1} in Fig. 5b.

In addition to the atypical responses to Pb^{2+} and Se(IV), sodium dodecylsulfate did not induce toxic responses for *E. coli* at any tested concentration. Rather, samples showed increased metabolic activity in the presence of SDS, potentially indicating it is being utilised

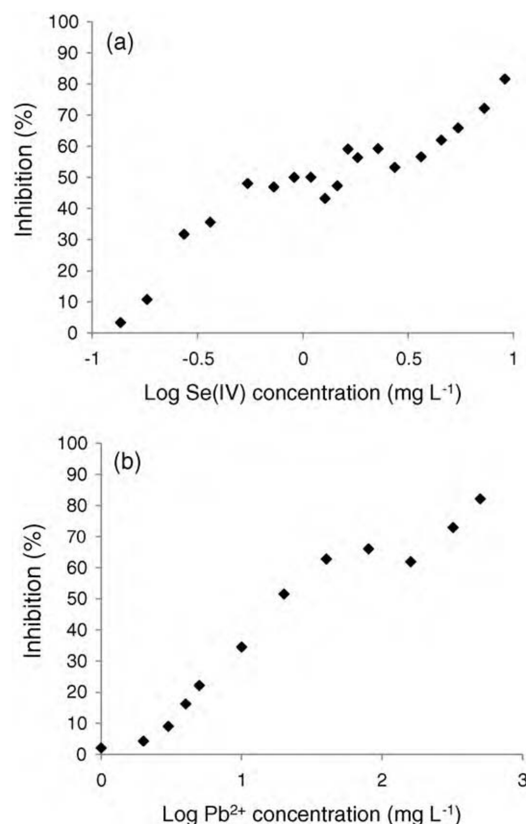

Fig. 5. Non-standard concentration-response curves for (a) selenium (IV) and (b) Pb^{2+} using the optimised FM-TOX assay with *E. coli*. Note log transformation applied on concentration (x) axis.

as a substrate by the *E. coli*. The membrane characteristics of *E. coli* offer protection from detergents [33], and this resistance is exploited in selective growth media for the isolation and enumeration of *E. coli*. So, FM-TOX with *E. coli* would be unsuitable for the detection of detergents in real samples. However, resistance to SDS may be a useful characteristic in real-world applications. For example, in municipal wastewater toxicity testing, high concentrations of surfactants are a regular occurrence, but do not necessarily indicate a problematic event for the treatment facility as the activated sludge community has adapted to cope with biodegradable surfactants [25]. Therefore, a toxicity test insensitive to surfactants would provide more operationally useful data on the potential impacts of influents on treatment efficiency.

One major advantage of FM-RES bioassays is their ability to utilise different microbial species as the biocomponent, unlike the MicroTox® test. Morris et al. [5] listed a range of 25 microbial species capable of using ferricyanide as a mediator, and previous FM-TOX studies have utilised *E. coli*, *K. oxytoca*, *B. subtilis* and *P. putida* [15,18]. Some differences in toxic response were observed, but of the bacteria listed, only *E. coli* has been tested against a range of toxicant classes. The other three species have been tested with chlorinated phenols only and did not display dramatically different results.

The use of different or multiple bacterial species in the FM-TOX test offers a promising avenue for customisation of the assay for a particular toxicity profile. For example, as described above, the membrane characteristics of *E. coli* offer it some protection from SDS or Pb^{2+}, but other bacteria may not respond in the same manner. The use of a mixed microbial consortium was found to obtain more consistent results for BOD testing [10], and such an approach could be considered in addition to the investigation of different single-species biocatalysts for FM-TOX. Investigation of the responses of differing bacterial strains to a variety of toxicant classes may yield FM-TOX assay configurations with improved sensitivity to specific toxicants of interest, or the ability to select a biocatalyst with a sensitivity range appropriate to a particular application. If multiple bacteria with differing profiles were used in conjunction with each other, the results may even enable identification of toxicant classes within a complex real sample.

The optimised FM-TOX configuration presented in this paper is tuned for sensitive determination of toxicity profiles for simple solutions containing toxicants. Toxicity testing of real samples, such as industrial wastewaters, presents a number of challenges on top of those posed by the determination of quantitative IC_{50} values. Wastewaters can contain complex mixtures of stimulatory and inhibitory compounds, but FM-based assays will only capture the net change in metabolism [12]. Therefore, the conditions of the FM-TOX assay, such as the relative durations of the exposure and measurement step, are likely to be even more influential in determining the outcome of a wastewater toxicity assay.

4. Conclusion

This paper describes a number of novel advances in the use of ferricyanide-mediated bioassays for toxicity assessment. The move to a two-step approach improves sensitivity and comparability across toxicant types, while eliminating temporary stimulatory effects induced by exposure to low concentrations of a toxicant. The exclusion of GGA from the test mixture also improves sensitivity, comparability and enables the reporting of IC_{50} results derived from inhibition of total metabolism, rather than inhibition of a fraction of the metabolism as in previous FM-TOX assays. Toxicity test results displayed classic sigmoid-shaped curves for a range of toxicants, including chlorinated phenols, heavy metals and other inorganic species. However, there were also compounds tested that induced atypical inhibitory effects on *E. coli*. The expansion of the assay to include other types of bacteria may allow enhanced specificity for particular applications, as well as greater sensitivity or resistance to specific toxicants or toxicant classes.

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