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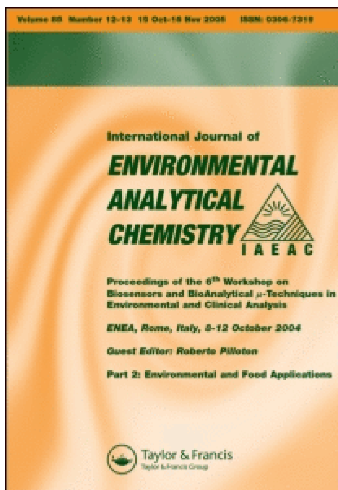

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Toxicity assessment of chlorophenols using a mediated microbial toxicity assay

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Toxicity assessment of chlorophenols using a mediated microbial toxicity assay

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While direct toxicity assessment (DTA) is now widely recognised as a useful tool for environmental risk assessment, many existing tests fail to meet end-user needs. This article describes the significant progress made to the MICREDOX® DTA assay, developed at Lincoln Ventures Ltd, brought about by miniaturising this assay to a multi-well plate format combined with limiting current microelectrode transduction. The benefits have been reduced: preparation time, reduced assay time, lower material costs and a higher level of replication achieved. To validate the precision of the miniaturised format, the concentrations required to cause a 50% decrease in signal (EC_{50}) by an archetypal group of toxicants, the chlorophenols, were determined using two terrestrial bacterial strains, *Escherichia coli* K12 and *Klebsiella oxytoca* 13183. The assay time was then reduced by stepwise adjustment of the incubation time, from 60 down to 5 min, and the EC_{50}s reported by *E. coli* to each of the toxicants after 45, 30, 15 and 5 min incubations were determined. The results obtained match closely with those reported by the Activated Sludge Respiration Inhibition Test and confirm the miniaturised multi-well plate MICREDOX® DTA assay reliably reports representative EC_{50} values for these toxicants. The previously described trends of increasing toxicity with increasing chlorine substitution and the observation that *meta*-substituted chlorophenols are more toxic than their *ortho*-substituted counterparts are also confirmed. The ability to monitor toxicity using terrestrial organisms, in volumes amenable to multi-well microtitre plates and incubations requiring only a few minutes, facilitates the rapid generation of highly reproducible, easy to operate and inexpensive DTA measurements.

Keywords: direct toxicity assessment; microelectrode amperometry; chlorophenols; *Escherichia coli*; *Klebsiella oxytoca*

1. Introduction

There has been intense activity into microbial-based assays in recent times. This stems from demands by the pharmaceutical industry, homeland security and environmental sector for assays that detect chemicals of interest and that also assess the consequences of exposure to those chemicals or biological agents. Microbial cells offer a range of response

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mechanisms following exposure to biological toxins and an increased understanding of these biological pathways over the past 20 years, coupled with technological advances in micro-fabrication, have accelerated the production of a range of diagnostic devices for environmental contaminants.

One area of application for direct toxicity assessment (DTA) testing is the assessment and regulation of complex chemical effluents at their point of discharge and for reporting the quality of receiving waters [1,2]. Currently, many bioassays that monitor toxicity levels of compounds present in water incorporate representatives from plants, invertebrates and fish as the sensing agents [3,4]. Unfortunately, many of these tests are time consuming, expensive and are no longer ethically acceptable, and there is a move towards using the simplest practical forms of life as the test organism [5,6]. Hence, using microorganisms for DTA testing is attractive: the tests can be based on a broad range of responses, are fast, less labour intensive and less expensive than testing with higher trophic level indicators. Toxic effects can, however, arise from different modes of action and therefore cannot be properly assessed from any single assay; a battery of test methods representative of different trophic levels is recommended [7]. Rapid DTA methods based on mediated-microbial assays offer a number of advantages, in comparison to other microbial-based assays, since they are amenable to optical and electrochemical techniques and, generally, there is no specific requirement for particular microbial species. This allows single species, or a mix of species, to be selected that are appropriate to the environment being monitored.

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Mediated microbial assays use artificial redox compounds (mediators) to intercept electrons exchanged during cellular catabolic redox processes and the change in redox state of the mediator is a readily quantifiable index of cellular catabolism. Tizzard [8] reported a rapid mediated microbial-based assay, MICREDOX®, for determining the DTA of toxicants. This assay, originally designed to rapidly assess the biochemical oxygen demand (BOD) of wastewater [9,10], was modified to monitor the impact of toxicants on living microorganisms. The MICREDOX® assay is characterised by high levels of biocatalyst (whole microorganisms) and synthetic mediator (potassium hexacyanoferrate), which together facilitate a fast reaction whereby the microbial oxidation of organic substrate is coupled to the reduction of the redox mediator. Throughout the 60 min incubation, reduced mediator accumulates as a by-product of bacterial respiration and this registers the amount of bioconversion that has occurred. After the incubation, the quantity of the reduced mediator is measured using electroanalytical techniques to give a direct measurement of the microbial respiratory activity [9]. When MICREDOX® is performed as a DTA assay, the electrochemical signal generated from the re-oxidation of microbially reduced mediator (produced by healthy cells) is compared to the signal produced by cells that have been subjected to a fixed level of toxicant. The ratio of the electrochemical signal, recorded in the presence of the toxicant relative to that recorded in the absence of toxicant, provides an index of the respiration inhibition (Inhibition Quotient, IQ).

To evaluate the capability of this bioassay to perform rapid DTA measurements, Tizzard [8] tested a variety of terrestrial bacterial strains with the reference compound, 3,5-dichlorophenol (3,5-DCP). They demonstrated that the MICREDOX® DTA biotoxicity assay generated sensitive and reproducible data and was well suited for rapid toxicity assessment. Moreover, measuring the re-oxidation of mediator using limiting-current microelectrode amperometry, in place of bulk electrolysis, produced significant advances in terms of simplicity, reliability and reproducibility and also facilitated real time

analysis generating data that showed the development of toxic responses [10]. Tizzard [8] concluded there was no analytical advantage in extending the incubation of microorganisms and 3,5-DCP beyond 60 min, with the toxic response clearly evident in the limiting-current profile after only 15 min.

In this article, we report the procedures for, and advantages gained by, miniaturising the MICREDOX® DTA assay to a final volume of 200 µL and the effect of reducing the incubation time from 60 to 5 min. The reduction in assay size has reduced the cost of the DTA assay, and most significantly, a larger number of individual assays (up to 96) can be performed simultaneously. The reduction in incubation time speeds up the assay, increasing the prospect of this assay providing a response within a time-frame that meets the needs of the wastewater industry for an upset early warning device [11]. Data are presented that compares the miniaturised DTA MICREDOX® assay with other commercially available DTA assays such as Microtox® and ToxAlert and with its larger-scale predecessor. EC_{50} values for a range of chlorophenols are reported and these are compared with published data derived from other commonly used microbially based DTA assays.

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2. Experimental

2.1 *Chemicals*

All chemicals were of analytical grade and purchased from Sigma-Aldrich, St Louis, USA. The five toxicants selected for this study were pentachlorophenol (PCP), 3,5-DCP, 2,4-dichlorophenol (2,4-DCP), 4-chlorophenol (4-CP) and phenol (P).

All reagents used were prepared gravimetrically using a Mettler AE200 balance (Toledo, Switzerland) and made up to volume with sterile distilled water at room temperature. All chlorophenol stock solutions were wrapped in aluminium foil and stored at 4°C. Potassium hexacyanoferrate (III) preparations (0.25 M) were wrapped in aluminium foil and stored at room temperature in the dark. Prior to commencing the assay, all reagents excluding the toxicant, were sparged with O_2-free N_2 for 15 min in a 37°C water bath.

2.2 *Bacterial cultures*

Escherichia coli K12 (*E. coli*) was purchased from the New Zealand Reference Culture Collection, Medical Section (ESR); *Klebsiella oxytoca* 13183 (*K. oxytoca*) was purchased from the American Type Culture Collection (ATCC).

K. oxytoca and *E. coli* were maintained on Difco m-Plate Count agar plates at 4°C and re-plated weekly to ensure fresh cultures. All pre-cultures were grown in ½ Difco m-Plate Count agar broth for 16 h on an orbital shaker at 200 revolutions per minute (rpm). *K. oxytoca* and *E. coli* were pre-incubated at 30 and 37°C, respectively. Pre-cultures were stored at 4°C until required.

K. oxytoca overnight cultures were grown in basal salts medium (Na_2HPO_4, 4gL^{-1}; K_2PO_4, 2gL^{-1}; $(NH_4)_2SO_4$ 1.0gL^{-1}) plus 2 mL of Herbert's trace elements solution [12], supplemented with 10 mM sodium succinate. *E. coli* overnight cultures were grown in Davis minimal media [13]. A 1% inoculum of pre-culture was added to the appropriate media and grown for 16 h, to late stationary phase, on an orbital shaker at 200 rpm held at 30°C for *K. oxytoca* and 37°C for *E. coli*.

2.3 *Bacterial cell preparation*

Cells were harvested using an Eppendorf 5810 centrifuge at 11,000 rpm at room temperature. The resultant cell pellet was washed three times in phosphate buffer (KH_2PO_4 10.62 mg L^{-1}, K_2HPO_4 21.25 mg L^{-1}) and spun at 11,000 rpm at room temperature between washes. The washed pellet was re-suspended in phosphate/saline buffer (KH_2PO_4 10.62 mg L^{-1}, K_2HPO_4 21.25 mg L^{-1}, KCl 7.46 mg L^{-1}). The cell concentration was adjusted to an absorbance value of 25.0, as measured at 600 nm using a UNICAM 8625 UV/VIS spectrophotometer. The cell suspensions were deoxygenated by sparging with O_2-free N_2 for 15 min before use.

2.4 *MICREDOX® toxicity assay*

Each of the five toxicants was tested, in triplicate, at up to eight different concentrations for each bacterium. The miniaturised assays were performed in Sarstedt 96-well microtest plates with each assay containing 44 µL of 0.25 M potassium hexacyanoferrate (III), 119 µL of substrate solution (glucose 75 mg L^{-1}, glutamic acid 75 mg L^{-1}, GGA) spiked with toxicant at the appropriate level and 37 µL of re-suspended cells ($OD_{600\,nm}$ 25); a total volume of 200 µL. The plates were flushed with O_2-free N_2, sealed with Axygen Seal Plate Sealing Film to maintain anaerobic conditions, wrapped in aluminium foil to exclude light and incubated on an orbital shaker at 200 rpm. The plates were incubated at 37°C for durations of either 60, 45, 30, 15 or 5 min. Assays were terminated by transferring the contents of each well into a 500 µL eppendorf tube and centrifuging at 10,000 rpm for 5 min to pellet the microbial cells. The supernatants were removed and placed into clean eppendorf tubes, covered with O_2-free N_2 and stored at 4°C until microelectrode amperometry analysis could be performed.

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2.5 *Control incubations*

Control incubations refer to incubations that contained de-ionised water in place of the toxicant; positive and negative controls refer to the presence or absence of GGA substrate, respectively.

2.6 *Microelectrode amperometry signal detection*

Limiting current microelectrode amperometry was used to measure the quantity of reduced mediator produced during the microbial incubation. A 25 µm Pt electrode was cleaned by sonicating in distilled water for 3–5 min and then polished on a flocked twill pad (Leco Corporation, St Joseph, Michigan, USA) for 20 s using a slurry of 0.3 µm α-alumina powder (Leco Corporation, St Joseph, Michigan, USA) in distilled water. The electrode was finally rinsed with distilled water and excess water carefully removed by touching the edge of the electrode with lint-free tissue. The Ag/AgCl reference (BAS MF-2079) and auxiliary electrodes (BAS MF-1032) were directly immersed in phosphate saline buffer supporting electrolyte contained within a cell vial (BAS MF-1082). A 150 µL aliquot of the supernatant was confined in a separate working-electrode compartment (BAS MF-2031, 2 mL minicell). The working-electrode was poised at +450 mV relative to the reference electrode and the anodic current obtained 10 s after imposition of the applied potential was taken as the limiting current value. After each analysis, the

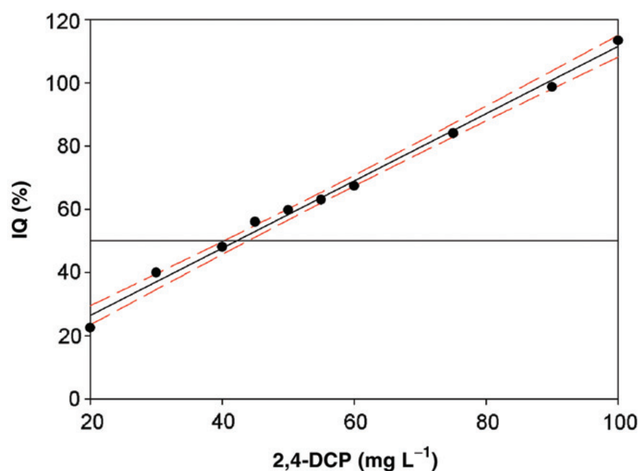

Figure 1. Inhibition profile (60 min) to 2,4-DCP obtained using *E. coli*.

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working-electrode was re-polished and the BAS MF-2031 minicell rinsed in distilled water and dried using lint-free tissue.

For each toxicant concentration, the microelectrode amperometric readings were converted to an IQ by applying the following equation:

$$\mathrm{IQ} = 100 \times \left[1 - \left(\frac{i_{\mathrm{lim}}(\mathrm{sample}) - i_{\mathrm{lim}}(c_{-})}{i_{\mathrm{lim}}(c_{+}) - i_{\mathrm{lim}}(c_{-})}\right)\right] \tag{1}$$

In Equation (1), i_{lim}(sample) is the amperometric limiting-current (i_{lim}) for a sample spiked with toxicant, $i_{\mathrm{lim}}(c_{-})$ is the limiting current of the negative control and $i_{\mathrm{lim}}(c_{+})$ is the limiting current recorded for the positive control.

The IQ calculated at each toxicant concentration was graphed against its corresponding concentration on either a logarithmic or a linear scale using SigmaPlot 9.0 (Systat Software, San Jose, USA). A best-fit regression line was fitted against the linear section of the resulting curve to determine the EC_{50} values. The EC_{50} values were interpolated from the intersection of the best-fit regression line with the 50 percentile IQ (Figure 1). Confidence intervals of 95% (95% CI) were determined using SigmaPlot 9.0.

3. Results and discussion

3.1 *Limiting currents measured after an incubation*

The concentration of microbially reduced potassium hexacyanoferrate (II) accumulating over the incubation stage provides the analytical signal. Examples of the limiting currents obtained with *E. coli*, when incubated for 60 min and spiked with various concentrations of 2,4-DCP subsequent to the incubation step, are shown in Table 1. To perform the range of measurements presented in Table 1 in triplicate requires 30 of the 96 wells, thereby allowing the assessment of up to three toxicants per plate in this manner.

As expected, in Table 1 the maximum signal corresponds to the positive control (C_{+}) and the presence of toxicant registers a dose-dependent response seen by the attenuation of the limiting currents recorded at the 25 μm Pt microelectrode.

3.2 EC_{50}s derived from MICREDOX® DTA assay after a 60 min incubation

Having obtained the limiting currents for 2,4-DCP with *E. coli*, as shown below in Table 1, the IQs at each toxicant concentration were calculated using Equation (1) and plotted on a linear or semi-logarithmic scale. The decision on which scale to use was decided based on the goodness-of-fit (r^2). The toxicant EC_{50} concentrations and 95% CIs were read off as shown in Figure 1.

Toxicity assays are not absolute and report an index relative to an unaffected control, thus the magnitude of their response is bench-marked by the use of reference toxicants. Of the families of chemical substances available, the chlorophenols are commonly used as reference toxicants and the OECD 209 Activated Sludge Respiration Inhibition Test (ASRIT) [14] also advises the use of 3,5-DCP as a reference toxicant. The collective EC_{50} results for the various combinations of bacteria and toxicant, compiled from experiments performed using two bacteria (*E. coli* and *K. oxytoca*), incubations of 60 min and five toxicants (PCP, 3,5-DCP, 2,4-DCP, 4-CP and P) are summarised in Table 2.

Table 1. Limiting currents for *E. coli* incubations (60 min) with controls and 2,4-DCP.

2,4-DCP ($mg\,L^{-1}$)	i_{lim} (nA)[a]	IQ[b]
C_+[c]	33.8 ± 0.9	
20	29.0 ± 1.0	22
30	25.2 ± 0.7	40
40	23.5 ± 1.1	48
45	21.7 ± 1.3	56
50	20.9 ± 0.7	60
55	20.2 ± 1.2	63
60	19.3 ± 0.8	67
75	15.7 ± 0.6	84
90	12.5 ± 0.6	99
100	9.4 ± 1.0	113
C_-[d]	12.2 ± 0.7	

Notes: [a]Error = S.D. of duplicates.
[b]Calc. from Equation (1).
[c]C_+ = positive control.
[d]C_- = negative control.

Table 2. MICREDOX® (60 min) EC_{50} values for chlorophenols.

	EC_{50} ($mg\,L^{-1}$)[a]	
Toxicants	*E. coli*	*K. oxytoca*
P	229 (216–240)	n.d.[b]
4-CP	175 (134–220)	139 (110–170)
2,4-DCP	42 (40–44)	50 (38–70)
3,5-DCP	5.8 (5.0–7.5)	1.3 (0.65–11)
PCP	2.1 (1.8–2.3)	1.1 (0.3–2.0)

Notes: [a]95% CI in parentheses.
[b]Not determined.

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The miniaturised MICREDOX® format was initially appraised using 3,5-DCP with the microorganisms *E. coli* and *K. oxytoca*. Table 2 reports the 60 min EC_{50} values to 3,5-DCP obtained with *E. coli* and *K. oxytoca* as 5.8 and 1.3 mg L^{-1}, respectively. The valid range for the EC_{50} (3 h) of 3,5-DCP using ASRIT is between 5 and 30 mg L^{-1} [14]. While the EC_{50} value obtained for *K. oxytoca* falls outside the valid range prescribed by the activated sludge respiration test [14], overlap in their respective 95% CIs (5.0–7.5 mg L^{-1} for *E. coli* and 0.65–11 mg L^{-1} for *K. oxytoca*) was observed. Previously, Tizzard [8] tested the toxicity of 3,5-DCP with three terrestrial bacterial strains, *E. coli*, *Pseudomonas putida* and *Bacillus subtilis*, using the larger-scale MICREDOX® assay, and reported EC_{50} (60 min) values of 7.0, 8.0 and 7.5 mg L^{-1}, respectively. The larger-scale MICREDOX® assays contained a total volume of 16.26 mL, with reagent volumes of approximately 100-fold greater than for the miniaturised format. Despite the introduction of an additional terrestrial microorganism, *K. oxytoca*, and replacement of N_2-sparging by N_2-flushing and shaking to accommodate a multi-well plate format, the similarity in EC_{50} values derived using both the larger-scale and miniaturised MICREDOX® formats demonstrates the resilience of assay.

Four additional toxicants, P, 4-CP, 2,4-DCP and PCP, were investigated in order to demonstrate that the DTA MICREDOX® assay could report robust and meaningful EC_{50} values that matched the response pattern in published data [15].

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Among the five toxicants tested in this study, P was the least toxic and Table 2 reports the MICREDOX® (60 min) EC_{50} (*E. coli*) value to P as 229 mg L^{-1}. EC_{50} determinations for the four chlorophenol toxicants were made separately with two terrestrial bacteria, *E. coli* and *K. oxytoca*. Table 2 reports the MICREDOX® 60 min EC_{50} values to 4-CP obtained with *E. coli* and *K. oxytoca* as 175 and 139 mg L^{-1}, respectively. An overlap of the 95% CIs for the two microorganisms was observed (134–220 mg L^{-1} for *E. coli* and 110–170 mg L^{-1} for *K. oxytoca*).

Table 2 reports the MICREDOX® 60 min EC_{50} values to 2,4-DCP obtained with *E. coli* and *K. oxytoca* as 42 and 50 mg L^{-1}, respectively. The 95% CIs for *E. coli* and *K. oxytoca* are 40–44 and 38–70 mg L^{-1}, respectively. Both these values are higher than those determined for 3,5-DCP, indicating that 3,5-DCP is the more toxic of the two structural isomers.

As expected, the fully chlorine-substituted PCP was the most toxic of this group of toxicants and Table 2 reports the MICREDOX® 60 min EC_{50} values to PCP obtained with *E. coli* and *K. oxytoca* as 2.1 and 1.1 mg L^{-1}, respectively. An overlap of the 95% CIs for the two microorganisms was observed (1.8–2.3 mg L^{-1} for *E. coli* and 0.3–2.0 mg L^{-1} for *K. oxytoca*).

3.3 *Effect of incubation time*

Previous work [8] demonstrated that the toxic response was clearly evident in the limiting-current profile after only 15 min, but there was insufficient data from that study to determine the EC_{50} values at incubations less than 60 min. Therefore, in order to assess the effect of decreasing the incubation times on the EC_{50} values derived, the incubation time was reduced stepwise from 60 down to 5 min. The EC_{50} values reported by *E. coli* to 2,4-DCP after 5, 15, 30, 45 and 60 min incubations are shown in Figure 2 along with a best-fit linear regression line.

In Figure 2, as the exposure time was progressively shortened the apparent EC_{50} (*E. coli*) value to 2,4-DCP increased, i.e. the toxic impact of 2,4-DCP decreased. The EC_{50}

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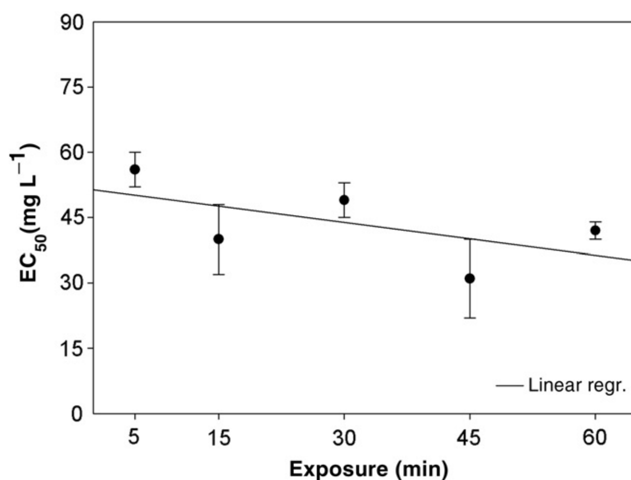

Figure 2. Effect of exposure time on 2,4-DCP EC_{50} values using *E. coli*.

Table 3. Effect of exposure time on EC_{50} values.

	EC_{50} (mg L^{-1})[a] at various exposures (min)				
Toxicant (species)	5	15	30	45	60
P					
(*E. coli*)	250 (240–256)	254 (252–258)	269 (264–273)	234 (223–246)	229 (216–240)
(*V. fischeri*)	29.8[b]	34.2[b]	35.8[b]		
4-CP					
(*E. coli*)	176 (173–180)	203 (191–217)	207 (200–211)	195 (184–205)	175 (134–220)
(*V. fischeri*)	8.49[b]	9.10[b]	8.30[b]		
2,4-DCP					
(*E. coli*)	56 (53–60)	40 (32–46)	49 (46–53)	31 (22–38)	42 (40–44)
(*V. fischeri*)	4.70[b]	5.04[b]	5.52[b]		
3,5-DCP					
(*E. coli*)	21 (12–50)	13 (9–22)	16 (12–26)	9.2 (7.4–11.0)	6.1 (4.8–7.4)
(*V. fischeri*)	4.39[b]	3.73[b]	3.91[b]		
PCP					
(*E. coli*)	2.8 (2.6–2.9)	2.9 (2.8–3.0)	3.2 (3.0–3.4)	2.6 (2.3–2.7)	1.8 (1.5–2.0)
(*V. fischeri*)	0.92[b]	0.61[b]	0.52[b]		

Notes: [a]95% CI in parentheses.
[b]Ref. [16].

of 2,4-DCP was 42 mg L^{-1} after 60 min exposure whereas it was 56 mg L^{-1} after 5 min exposure. This response pattern to a toxicant is expected as it is liable to be both time and concentration dependence. In comparison to the divergence among the 2,4-DCP EC_{50} values reported in Tables 2 and 3 for various assays and biocomponents, the shift in apparent EC_{50} value with decreased exposure time is quite minor.

The EC_{50} values reported by *E. coli* in the MICREDOX® assay to each of the toxicants after 5, 15, 30, 45 and 60 min incubations are summarised in Table 3. A linear least squares fit of the data in Table 3 reveals that the trend of increasing apparent EC_{50} values as the exposure time in this assay is progressively shortened was upheld by each toxicant in this study. The changes to the respective EC_{50} values are not, however, of sufficient magnitude to nullify the advantage accrued by reducing the assay time down to minutes. Bioluminescent toxicity assays continuously monitor the reduction of bioluminescence throughout the exposure time and thus it is common for them to report EC_{50} data for 5, 15, and 30 min exposure. In order to compare the trends observed in this study, EC_{50} values to the five toxicants by the Microtox® assay [16] taken at 30, 15, and 5 min exposures are also included in Table 3. It is interesting to note that for both assays the EC_{50} for 4-CP remains relatively invariant with exposure time, the trend of increasing apparent EC_{50} values with shortened exposure time was common for 3,5-DCP and PCP, however for the toxicants P and 2,4-DCP, the Microtox® assay reported a reverse trend. For those toxicants, the Microtox® EC_{50} values decreased with shortened exposure time by 17 and 15%, respectively.

3.4 *Comparison of MICREDOX® DTA assay to other DTA assays*

Increasingly, DTA assays are recognised as useful tools for environmental risk assessment and for monitoring the discharge of complex chemical effluents into receiving waters. This has stimulated the development and testing of assays that are faster and less expensive than those previously reliant on higher trophic indicators. In Table 4, the results reported by the MICREDOX® DTA assay are compared to other commonly used microbially based DTA assays: sub-mitochondrial particles (SMP) [15], Microtox® [16,17], ToxAlert® [18], CellSense [19] and ASRIT [20].

These assays are all based on the inhibition of respiration and, with the exception of the SMP assay, use whole bacterial cells. Toxic or inhibitory substances modify bacterial respiration, which in turn modifies oxygen consumption and so the quantity or the rate of oxygen depletion is an indication of whether an effluent is biodegradable (i.e. non-toxic), inhibitory or toxic. Microbial respiration, and its inhibition by various environmental pollutants, can be measured both optically and electrochemically. The SMP assay optically monitors the rate of NADH production. A comprehensive study using SMPs that evaluated the toxicity of 14 chlorophenols relative to their structural characteristics, reported an EC_{50} (30 min) for 3,5-DCP at 0.49 mg L^{-1} [15]. This value reflects the higher sensitivity to the toxicant by the SMP assay relative to its whole cell counterpart and its EC_{50} for 3,5-DCP is outside the limits prescribed by OECD 209 [14]. The SMP assay was instrumental in establishing a direct relationship between toxicity and the chlorine content of the chlorophenols [15]. Given whole cell toxicity assays based on respiration and SMPs share a common bioresponse element, the electron transport chain, one could anticipate that a similar response pattern between toxicity and the chlorine content would also be evident in whole cell respiration inhibition assays, such as the MICREDOX® assay.

The Microtox® and Toxalert® assays incorporate bioluminescent *Vibrio fischeri*, with toxic responses being quantified by a change in the intensity of light emissions. The bioluminescent marine organism *V. fischeri* is also an Internationally Approved Toxicity Testing Standard (DIN 38412T34), and its EC_{50} to 3,5-DCP has been reported at 3.39 mg L^{-1} (30 min) in the Microtox® assay [17] and 6.17 mg L^{-1} (30 min) in the

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Table 4. Comparison of MICREDOX® (60 min) EC_{50} values to other DTA assays.

	DTA assay (EC_{50} mg L^{-1})					
Toxicants	SMP	Microtox (*V. fischeri*)	ToxAlert (*V. fischeri*)	MICREDOX (as listed)	ASRIT (activated sludge)	CellSense (as listed)
P		35.8 [16] 23.50 [24]	7.99 [27]	229 (*E. coli*)	783 [20]	
4-CP	10.94	8.30 [16] 42.7 [24]	21.21 [24]	175 (*E. coli*) 139 (*K. oxytoca*)	178 [20]	201 (*E. coli*) [3] 239 (*P. putida*) [19]
2,4-DCP	1.39 [15]	5.52 [16]	2.85 [27]	42 (*E. coli*) 50 (*K. oxytoca*)	49.5 [20]	393 (*E. coli*) [3] 247 (*P. putida*) [19]
3,5-DCP	0.49 [15]	3.91 [16] 13.4 [24]	6.17 [18]	5.8 (*E. coli*) 1.3 (*K. oxytoca*)	12.5 [20]	9.8 (activated sludge) [18]
PCP	0.08 [15]	0.52 [16]	1.07 [27]	2.1 (*E. coli*) 1.1 (*K. oxytoca*)	2.5 [20]	0.037 (*E. coli*) [3] 320 (*P. putida*) [19]

ToxAlert® assay [18]. These values for 3,5-DCP also fall within the region spanned by the 95% CI reported for *E. coli* and *K. oxytoca* in Table 2.

The rate of oxygen depletion, normally measured electrochemically with an oxygen electrode, is the basis of the analytical signal for the ASRIT assay. The MICREDOX® and CellSense assays electrochemically monitor the attenuation of microbially produced mediator (hexacyanoferrate (II)) after exposure to a toxic substance.

In Table 4, the assays are arranged horizontally in order of decreasing sensitivity to the toxicants (left to right) and vertically in order of the toxicant's toxicity. The SMP assay demonstrates the highest sensitivity to the toxicants with the EC_{50} for the standard, 3,5-DCP, at approximately one-tenth of the concentration reported by the other assays. The *V. fischeri* assays, Microtox® and ToxAlert®, are also more sensitive than ASRIT, probably because *V. fischeri*, a marine organism, is not represented in the activated sludge bacterial community [21,22]. Table 4 shows that MICREDOX® and ASRIT report similar EC_{50} values, with the MICREDOX® assay being marginally more sensitive overall. Given that all four bacteria so far tested in the MICREDOX® DTA assay, *E.coli*, *K. oxytoca*, *B. subtilis* and *P. putida*, are also found in activated sludge, it is no surprise to see that the sensitivity to the toxicants reported in Table 4 bears close resemblance to the values reported by the ASRIT assay [20]. CellSense is less consistent than the other assays and it is difficult to reconcile the differences arising when *E. coli* [3] is replaced by *P. putida* [19].

Previously, two general observations have been made regarding chlorophenol toxicity: that the toxicity of the chlorophenols increases with increasing chlorine substitution and that the *meta*-substituted isomer (3,5-DCP) is more toxic than the *ortho*-substituted isomer (2,4-DCP) [15]. The MICREDOX® DTA EC_{50} values presented in Table 4 support these observations, as do the SMP, Microtox® and ASRIT assays. However, ToxAlert® reports the *ortho*-substituted DCP isomer as more toxic than the *meta*-isomer, and CellSense reports 2,4-DCP as less toxic than 4-CP.

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3.5 *Evaluation of the MICREDOX® DTA assay to meet end-user needs*

Attributes considered necessary to meet end-user needs for ecotoxicity testing include precision, working time, ease of use, relevance and cost per test [17,23]. Amongst those listed in Table 4, the *V. fischeri* assays, Microtox® and ToxAlert®, are reputed to be the most reproducible and results from two inter-laboratory comparisons [24] using 3,5-DCP, report coefficients of variation ranging from 9–29%. For comparison, the MICREDOX® DTA data presented in Table 1 have a group coefficient of variation of 5%. Allowing for an incubation time of 5 min, plus time to set-up and terminate the assay, MICREDOX® DTA testing can be completed in less than 3 h. Most of this at present arises due to the need for centrifuging the samples to obtain a supernatant prior to the signal detection step. In comparison, the *V. fischeri* assays require a working time of 1.75 h [17]. Adapting the MICREDOX® DTA methodology to a multi-well plate format means that, except for the electrochemical detection step, the assay is performed with highly standardised components. While microelectroelectrode amperometry is not routine in many analytical chemistry laboratories, it is a simple, reliable and rapid method for measuring the concentration of electroactive species, such as hexacyanoferrate (II) [25].

One of the major uses for toxicity assays is as 'upset early warning devices' in wastewater treatment plants [23] and in assessing five rapid direct toxicity assays, Dalzell [17] concluded that the *V. fischeri* assays were too sensitive and therefore not relevant to

the activated sludge process. Given the similarity of the MICREDOX® response to ASRIT, it is highly likely that MICREDOX® is a relevant model for monitoring activated sludge susceptibility. In a recent comparative study of ToxAlert®, CellSense and Biolog plates [18], the main advantages of the Biolog plate method were found to be the low cost and the short time to load and read the plates. For a 96-well plate MICREDOX® DTA assay, we estimate the cost of the consumables, excluding the cost of the microoganisms, to be less than $US5.

4. Conclusions

The MICREDOX® DTA methodology has been adapted to a multi-well plate format resulting in significant advantages. Without any loss in turnaround time, the assay's throughput is increased substantially. One 96-well plate allows for two controls plus 30 toxicant samples to be tested in triplicate, whereas previously, two controls and only five toxicant samples in duplicate, could be tested at one time [8]. The increased throughput gives scope to examine a wide array of samples centred about the likely EC_{50} concentration for a given toxicant. Multiple replicates reduce the error of estimation and facilitate the calculation of 95% CIs. The resultant inhibition profiles, as shown in Figure 1, readily provide toxicity estimates and 95% CIs at other than the EC_{50}, such as EC_{20} and EC_{80}. Together, the lower material costs and increased throughput in the number of samples processed make for more effective toxicity screening at less cost.

The results reported by the MICREDOX® DTA in this and previous papers [8,26] show good reproducibility despite using microorganisms from different genera. The sensitivity of the MICREDOX® DTA assay shown to the five toxicants closely aligns with ASRIT's sensitivity and three of the organisms, *E.coli*, *B. subtilis* and *P. putida*, report EC_{50}'s for 3,5-DCP that closely align with each other and fall within the 5–30 $mg\,L^{-1}$ window of normality prescribed by the ASRIT [14]. Figure 2 and Table 3 show that for each toxicant meaningful EC_{50} values were reported as the exposure time was decreased, even to as little as 5 min. This places the MICREDOX® DTA among the fastest of the DTA assays. A trend of increasing apparent EC_{50} values as the exposure time in this assay is progressively shortened was upheld by each of the five toxicants. Although it is unwise to generalise from a limited dataset such as Table 4, both assays (MICREDOX® and Microtox®) report greater variation in the apparent EC_{50} values with shortened exposure times as the toxicity of the toxicant increases. Given that 2,4-DCP's variation of EC_{50} with exposure is less than 3,5-DCP suggests that 2,4-DCP might be a better standard toxicant than 3,5-DCP.

Current development work on this assay is focussed toward the use of multi-well plates pre-loaded with freeze-dried microbial cells and at performing simultaneous measurements of the electrochemical signals directly within each well of a microtitre plate. The ability to perform multiple electrochemical measurements directly within the wells of a plate eliminates the centrifugation step and will substantially reduce the working time, down from 3 h to around 10–15 min, to complete the assay.

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