Lux-biosensor assessment of pH effects on microbial sorption and toxicity of chlorophenols

Gillian M. Sinclair a,*, Graeme I. Paton a, Andy A. Meharg b, Ken Killham a

a Department of Plant and Soil Science, University of Aberdeen, St. Machar Drive, Aberdeen AB24 3UU, UK
b ITE Monks Wood, Abbots Ripton, Huntingdon PE17 2LS, UK

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Abstract

Lux-marked bacterial biosensors and a commercial toxicity testing bacterial strain (Microtox®) were exposed to 2,4-dichlorophenol (DCP) and the light output response measured. Increasing DCP concentrations caused a decrease in light output in all three biosensors with an order of sensitivity (in terms of luminescence decrease over the DCP concentration range) of Pseudomonas fluorescens < Escherichia coli < Microtox®. Adsorption of DCP to E. coli was measured using uniformly ring labelled [14C]DCP and found to be very rapid. The effect of pH on toxicity and adsorption was also investigated. Low pH values increased the amount of DCP adsorbed to the cell and increased the toxicity of DCP. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Biosensor; 2,4-Dichlorophenol; Toxicity; Microbial sorption

1. Introduction

Whole cell microbial biosensors offer a powerful new approach to environmental monitoring, providing an indication of pollutant bioavailability, rather than the total concentrations obtained by traditional analytical techniques [1]. Biosensors are also able to monitor very low levels of chemicals, can work in complex matrices and have very fast response times [2]. Lux-marking of terrestrial bacteria offers scope for environmentally relevant, whole-cell biosensors which can either be toxin specific or general indicators of pollutant toxicity. Biosensors have been used to monitor environmental contamination by heavy metals [3] and organic contaminants [4], as well as toxicity in soils and water contaminated by industrial effluents [5], BTEX compounds [6] and chlorinated aromatics [7]. Lux-marked biosensors offer greater environmental relevance for luminescence-based testing in terrestrial systems compared to the Microtox® test which utilises the response of the naturally luminescent marine bacterium Vibrio fischeri [7].

The compound 2,4-dichlorophenol (2,4-DCP) was chosen for this study as it is a ubiquitous pollutant in the environment. Chlorophenols enter through several pathways, for example through use as herbicides, fungicides and general biocides [8] and their major use as preservatives for wood, textiles and
leather [9]. Chlorophenols also occur as the breakdown products of other pesticide compounds such as phenoxyacetic herbicides [9]. Pulp mill effluents, disinfection of water supplies by chlorine [9] and sewage sludge are all sources of chlorophenols in the environment [10]. Phenolic compounds exert a toxic effect on microorganisms by disrupting energy transduction either by uncoupling oxidative phosphorylation or by inhibiting electron transport [11]. Substituted phenols act by destroying the electrochemical proton gradient by transporting protons back across the membrane and/or inhibiting electron flow by binding to specific components of the electron transport chain [11]. As the luminescence of lux-marked bacterial biosensors depends on the products of the electron transport chain for light production, inhibition of this process due to chlorophenol toxicity will result in a decrease in light output. The aim of this study was to investigate the effect of pH on the toxicity and adsorption of 2,4-DCP to lux-marked biosensors.

2. Materials and methods

2.1. Bacterial strains

The bacterial strains used contained the V. fischeri luxCDABE genes. The lux-marked strains used in this set of experiments were Escherichia coli HB101 pUCD607 [12] and Pseudomonas fluorescens pUCD607 [13] which contained the genes as multicopy plasmids. All the cultures were freeze-dried and resuscitated for 2 h in 10 ml of LB medium (Luria-Bertani) at 25°C in an orbital shaker. E. coli was washed twice in 0.1 M KCl and P. fluorescens in 1 M KCl.

2.2. Toxicity testing of 2,4-DCP

E. coli and P. fluorescens were all exposed to a range of concentrations of 2,4-DCP. A 100-μl aliquot of washed cells was added to 900 μl of a dilution of 2,4-DCP ranging from 0 to 175 mg l⁻¹ in a 1-ml luminometer cuvette. The cells were added at an interval of 20 s between each cuvette and were read after 15 min, in a Bio-orbit 1251 luminometer with a Multiuse software package (Ver. 1.1). A vial of commercial toxicity testing bacteria, Microtox® (V. fischeri), was resuscitated in 2% NaCl at a temperature of 4°C and used immediately. A series of dilutions of 2,4-DCP ranging from 0 to 11.64 mg l⁻¹ was adjusted to 2% NaCl. The cells were then added to the dilutions as before and read in the luminometer after 15 min.

2.3. Adsorption of [¹⁴C]2,4-DCP by E. coli

The ¹⁴C-labelled 2,4-DCP used was purchased from Sigma and was uniformly ring labelled with a specific activity of 2.96×10⁸ Bq mmol⁻¹. A 100-μl aliquot of washed cells was placed in a plastic microcentrifuge tube and 250 μl of [¹⁴C]2,4-DCP solution (0.4 ml stock containing 17.53 μg ml⁻¹ [¹⁴C]2,4-DCP and 3.6 ml distilled water) was added. The cells were then incubated for a period between 0 and 76 min. The cells were spun down at 9447 g in a MSE Microcentaur microcentrifuge, the supernatant removed and 1 ml of a saturated solution of ice cold 2,4-DCP added. The cells were shaken for 2 min on ice (to maintain the low temperature of the cells and the 2,4-DCP) on an Ika Vibrax orbital shaker set at a motor speed of 1000. This sequence of centrifugation and 2,4-DCP addition was then repeated to allow determination of cell bound 2,4-DCP. The supernatant was removed and a 500-μl aliquot of tissue solubiliser (hydroxide of Hyamine 10-X (Packard)) was added to the cells. The cell pellet was dispersed and 500 μl of the resulting supernatant was placed in a scintillation vial with 2 ml of Hionic-fluor® (Packard). The ¹⁴C activity of the samples was determined using a Tri-carb liquid scintillation analyser-2500 TR using a standard ¹⁴C counting window. Each time period was measured in triplicate and blank samples without any cells were also measured to allow for any carry over of activity.

2.4. Cell number determination

A 100-μl aliquot of washed E. coli cells was placed in a microcentrifuge tube and 3 μl of green fluorescent nucleic acid stain in dimethylsulfoxide (LIVE/DEAD® Baclight®) added. The microcentrifuge tube was vortexed and stored in the dark for 15 min. A 10-μl aliquot of the mixture was placed in a Helber bacteria counting chamber (Fisher) with
2.5. Effect of pH on adsorption of [14C]2,4-DCP by E. coli

A solution of [14C]2,4-DCP (5.26 µg ml⁻¹) was adjusted to a pH of 4, 6 or 8 using HCl and NaOH. A 1000-µl aliquot of this solution was added to 50 µl of cells which were incubated for 2 min. The cells were spun down and the supernatant removed. A 250-µl aliquot of a saturated 2,4-DCP solution was also added to the corresponding pH value. The cells were shaken for 2 min and spun down for 3 min at 9447 g in a MSE Microcentaur centrifuge. The resulting supernatant was removed and the sequence repeated. A 500-µl aliquot of tissue solubiliser was added to the cell pellet and the pellet dispersed. The resulting solution was removed and added to 2 ml of Hionic-fluor® in a scintillation vial and read as before.

2.6. Effect of pH on toxicity of 2,4-DCP to E. coli

A saturated solution of 2,4-DCP was adjusted to the required pH by the addition of HCl and NaOH. Double deionised water was also adjusted to the required pH in the same way. A dilution series of 2,4-DCP was created using the pH adjusted water and 2,4-DCP to give a series ranging from 0 to 29.1 mg l⁻¹. This procedure was carried out to give the following pH values: 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5. Washed E. coli cells were added at 20-s intervals and the light output was measured after 15 min in a Bio-orbit 1251 luminometer as before.

2.7. Statistical analysis

One way ANOVA (analysis of variance) was carried out on all results using untransformed data and Minitab (Ver. 12). A pooled least significant difference (LSD) was calculated for Tables 1 and 2.

3. Results and discussion

3.1. Toxic action of chlorophenols

The results of the toxicity testing (Table 1) were expressed as EC values which represents the amount of 2,4-DCP required to reduce light output by a given value (either 50% or 25%). The EC₅₀ values were calculated by plotting a graph of bioluminescence as a percentage of the control (900 µl double deionised water and 100 µl cells) against 2,4-DCP concentration.

This study has shown that lux-marked biosensors show a toxic response to 2,4-DCP as represented by the low EC₅₀ values. Microtox® was shown to be the most sensitive with an EC₅₀ of 4.06 mg l⁻¹, with E. coli HB101 pUCD607 the most sensitive of the lux-marked biosensors used. The difference in sensitivity may partly be explained by the different ecological niches represented by the two organisms. V. fischeri, which is the basis of the Microtox® assay, is a marine organism whereas E. coli is a gut organism. The 2,4-DCP dilutions used in the toxicity testing had to be adjusted to 2% NaCl for the Microtox® assay. This may alter the mode of action or binding of the DCP to the cells.

The mode of toxic action of substituted phenols has been the subject of much research in the past few years. Substituted phenols, such as 2,4-DCP, cause toxic effects through a number of mechanisms which target cell constituents such as mitochondria and

<table>
<thead>
<tr>
<th>Biosensor</th>
<th>EC₅₀ (mg l⁻¹)</th>
<th>S.E.M.</th>
<th>EC₂₅ (mg l⁻¹)</th>
<th>S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas fluorescens</td>
<td>38.28a</td>
<td>8.45</td>
<td>34.18a</td>
<td>9.48</td>
</tr>
<tr>
<td>Microtox®</td>
<td>4.06b</td>
<td>0.54</td>
<td>1.47b</td>
<td>0.24</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>12.61b</td>
<td>4.120</td>
<td>11.64b</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Different letters denote significant differences (P ≤ 0.05).
bacterial cytoplasmic membranes. They can act in several ways as uncouplers, inhibitors or narcotic agents [14]. If it is possible for a weak acid to pass through the membrane in the anionic form, then it will act as an uncoupler [15]. Uncouplers have been shown to destroy the electrochemical proton gradient by transporting protons back across the cell membrane [11] at a rate faster than the respiratory chain or ATP synthase proton pumps [15]. It has also been proposed that chlorophenols may inhibit ATP synthesis due to an affinity of binding directly to components of the electron transport chain [11]. Substituted phenols can be structurally similar to reduced quinone molecules which are present at reasonably high concentrations in bacteria and carry electrons and proton equivalents [14]. Molecules which resemble quinones can bind to quinone binding sites blocking electron flow [14].

The decrease in light output would suggest that the metabolism of the cell is being disrupted by 2,4-DCP. This could be occurring either through the prevention of ATP synthesis, as ATP is required for light production, or by disrupting the electron transport chain.

The lux-marked E. coli not only proved to be the most sensitive lux-marked biosensor, but also showed a high activity (i.e. luminescence) across a wide range of pH values (across a pH range of 4.0–6.5, the relative light units (RLUs) did not fall below 21% of the maximum light output which was achieved at pH 5.5). Although Microtox® was shown to be sensitive to 2,4-DCP, it has also been shown to be responsive at a limited pH range and is therefore unsuitable for this study. Therefore E. coli was used in this study to examine the effect of pH on adsorption and toxicity.

3.2. Adsorption of 2,4-DCP

The results (Fig. 1) showed that there was a high capacity for adsorption of 2,4-DCP to the cell membrane. The rate of adsorption was very rapid at first, possibly due to the charged form of the 2,4-DCP. As the 2,4-DCP solution used in this experiment was not pH adjusted in any way, most of the molecules would have been in the non-ionised form, according to the pKₐ value of 7.68. Therefore, the 2,4-DCP would be in a highly lipophilic form and would be readily partitioned into the cell membrane [9] which may explain the very rapid uptake.

There have been few studies conducted on the adsorption of chlorophenols to bacterial membranes and most of the literature refers to pentachlorophenol (PCP). Adsorption studies with Mycobacterium chlorophenolicum PCP-1 using PCP over a range of concentrations and pH values have been performed [16]. PCP, being strongly hydrophobic, was easily incorporated into the hydrophobic cell membrane through binding with the side chains of mycolic acids in the cell wall. It is also possible that the PCP may be present in the lipophilic membranes of the cell [16].

3.3. Effect of pH on adsorption

pH had a major effect on the adsorption of 2,4-DCP by the cells (Table 2). The amount of adsorption to the cells increased with decreasing pH. At pH 4, there was a great deal more adsorption than at pH 8, where very little adsorption took place. Adsorption at pH 6 did not differ significantly from that at pH 4. The solution at pH 4 will have had the highest concentration of non-ionised 2,4-DCP molecules and therefore the most lipophilic molecules, whereas the pH 8 solution will have had the lowest concentration of non-ionised molecules.
and therefore will not have easily accumulated in the membrane.

The diffusion of the charged form of chlorophenol across the cell membrane appears to be the rate limiting step in the uncoupling process [14]. There are two possible forms of charged species, either a phenoxide or a heterodimer consisting of one phenol and one phenoxide but the forms present will depend on the pH of the chlorophenol solution [14]. It has been found that for phenols, the anionic form was less toxic than the protonised form (ionic) [17]. This may be due to electric charges present on the phenol molecules which hinder transport processes and binding to target sites.

It has been shown that pH had a strong effect on PCP adsorption by M. chlorophenolicum PCP-1 [16]. At low pH, particularly acid pH values, the adsorption capacity was increased, probably due to changes in the ionic and anionic forms. Reversible and irreversible adsorption of PCP occurred with irreversible binding due to the undissociated fraction of the PCP [16].

3.4. Effect of pH on the toxicity of 2,4-DCP

The pH of the 2,4-DCP solution greatly affected the toxicity of the solution to the cells as shown by the EC\textsubscript{50} values (mg l\textsuperscript{-1}) (Fig. 2) which were as follows: pH 4.0, 5.17; pH 4.5, 8.97; pH 5.0, 12.07; pH 5.5, 15.86; pH 6.0, 22.76; pH 6.5, 22.07. The EC\textsubscript{50} values were calculated by plotting the average bioluminescence of three bioassays as a percentage of the control (900 \( \mu \)l of pH adjusted double deionised water and 100 \( \mu \)l of cells).

A stimulation in light output occurred at low concentrations of 2,4-DCP possibly through one of two mechanisms. Lewis et al. [15] suggest that 2,4-DCP acts as an uncoupler causing protons to be transported across the membrane disrupting the proton gradient. The action of the cell in attempting to restore the gradient, may speed up electron transport. This would also increase the FMNH\textsubscript{2} (reduced flavin mononucleotide) respiration system increasing light output. However, Heitzer et al. [18] propose an alternative mechanism. The stimulation may be due to alterations in the fatty acid composition of the bacterial membrane due to changes in the synthesis patterns of the fatty acids. This may affect fatty acid supply and aldehyde formation for the light reaction causing an increase in light output.

As the pH fell, the 2,4-DCP appeared to become more toxic to the cells, shown by the decreasing EC\textsubscript{50} value. As shown in the previous results, the pH of the 2,4-DCP solution had a major effect on the adsorption of 2,4-DCP to the bacterial cells. These results, together with those from the pH adsorption experiment, suggest that if 2,4-DCP is not being adsorbed to the cell surface, then it cannot exert a toxic effect. At low pH, there was very high adsorption.

![Fig. 2. Effect of pH on the bioluminescence response of E. coli to varying concentrations of 2,4-DCP. The average bioluminescence of three bioassays was plotted. Key: pH 4, ●; pH 4.5, ■; pH 5, ▲; pH 5.5, ○; pH 6.0, □; pH 6.5, △.](image-url)

<table>
<thead>
<tr>
<th>pH</th>
<th>DCP adsorbed (mg/cell)</th>
<th>S.E.M.</th>
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<tbody>
<tr>
<td>4</td>
<td>34.33\textsuperscript{b}</td>
<td>4.78</td>
</tr>
<tr>
<td>6</td>
<td>25.66\textsuperscript{b}</td>
<td>6.71</td>
</tr>
<tr>
<td>8</td>
<td>1.28\textsuperscript{c}</td>
<td>0.52</td>
</tr>
</tbody>
</table>

\( ^{a} \)The mean number of metabolically active cells as determined by LIVE/DEAD\textsuperscript{®} Baclight\textsuperscript{®} in the assay solution was 2.65x10\textsuperscript{5} per \( \mu \)l. Different letters denote significant differences (\( P \leq 0.05 \)).
which correlates well with the very low EC\textsubscript{50} values found in this experiment.

The toxicity of chlorophenols to Microtox\textsuperscript{®} at various pH values has been tested [17] and it was found that toxicity decreased with increasing pH. They tested 2,4-DCP over a pH range covering the pK\textsubscript{a} of the molecule and found that the phenol form remained constant. They concluded that there was no contribution to total toxicity by the phenate form which must be either non-toxic or have very low toxicity compared to the phenol form.

4. Conclusions

This paper has demonstrated the toxicity of 2,4-DCP to the luminescence of \textit{lux}-marked \textit{E. coli} and \textit{P. fluorescens} and the naturally luminescent \textit{V. fischeri}. The toxicity was greatest at low pH, correlating with greatest observed adsorption of 2,4-DCP to the bacterial cell wall. \textit{Lux}-marked bacterial biosensors offer a powerful way forward for rapid screening of the effect of environmental variables on the fate and toxicity of pollutants.

Acknowledgments

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References