PERGAMON

Toxicon 38 (2000) 1203–1213

TOXICON

www.elsevier.com/locate/toxicon

Degradation of the cyanobacterial toxin cylindrospermopsin, from *Cylindrospermopsis raciborskii*, by chlorination

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Received 24 May 1999; accepted 14 September 1999

Abstract

Cylindrospermopsin, a potent cyanobacterial toxin produced by *Cylindrospermopsis raciborskii* and other cyanobacteria, is regularly found in water supplies of Queensland, Australia. This study focussed on the effectiveness of chlorination as a water treatment procedure for cylindrospermopsin degradation. The results demonstrate that relatively low chlorine doses (<1 mg l^{-1}) are sufficient for degradation of cylindrospermopsin, when the dissolved organic carbon content is low. However, if organic matter other than cylindrospermopsin is present in the solution, the effectiveness of chlorine for cylindrospermopsin degradation is reduced as other organic matter present consumes chlorine. Under the experimental conditions using samples with a solution pH of 6–9, a residual chlorine concentration of 0.5 mg l^{-1} was sufficient to degrade $>99\%$ of cylindrospermopsin. Toxin degradation via chlorination occurs within the first minute and no difference was observable between degradation in an open system and in a closed system. With a decrease of the pH from 6 to 4 a reduction in the efficiency of chlorine for degradation of cylindrospermopsin was observable, a possible indication that cylindrospermopsin is more stable to chlorine degradation at lower pH. However, in normal water treatment this is not relevant since the pH is consistently higher than 6. © 2000 Elsevier Science Ltd. All rights reserved.

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PII: S0041-0101(99)00210-X

1. Introduction

The list of toxin-producing cyanobacteria includes a number of organisms that are often present in natural waters. Cyanobacterial toxins have been associated with the poisoning of livestock, domestic pets and humans (Jochimsen et al., 1998; Pouria et al., 1998). In addition to acute effects, these toxins can also exhibit chronic effects and one group (the microcystins) exhibits tumour promotion activity (Ueno et al., 1996; Yu, 1994). Thus, the occurrence of cyanobacterial toxins in water has serious health implications.

Water supplied for public consumption should be maintained below guideline values for cyanobacterial toxins. The toxins that pose the greatest threat in drinking water are located within the cyanobacterial cell and are only released upon lysis of the cell wall (Berg et al., 1987). In order to render water safe for human consumption, it is necessary to remove both intact cells and also the toxins that have been released into the water. It is important to note that there are no guidelines currently set for the cyanobacterial toxin cylindrospermopsin.

Normal treatment processes (such as coagulation, flocculation and filtration) are ineffective in removing cyanobacterial toxins from water (Himberg et al., 1989). Activated carbon can however remove over 90% of a variety of different cyanobacterial toxins dissolved in the water. But the adsorbent is very expensive and has a finite life, which decreases with increasing organic matter in solution and the removal efficiency is affected by the type of carbon (Keijola et al., 1988; Lambert et al., 1996). More recently, oxidants such as chlorine and ozone have been proven effective for removal of cyanobacterial toxins. For example, effective removal of the peptide hepatotoxin microcystin has been achieved using a chlorine residual greater than or equal to 0.5 mg l^{-1}, at a solution pH <8 (Nicholson et al., 1994).

In South East Queensland, there have been several toxic cyanobacterial blooms in local water storages. North Pine Dam has been closed several times (1995 and 1996) due to an outbreak of *C. raciborskii* (Grant and Gubbels, 1996). The alkaloid hepatotoxin cylindrospermopsin is produced by *C. raciborskii*, a cyanobacterium regularly encountered in Queensland's reservoirs (Hawkins et al., 1997). A toxic strain of this organism was first isolated from Solomon Dam, which provides the source of drinking water for Palm Island (Queensland). In 1979, an outbreak of hepatoenteritis occurred amongst the community on the island, following treatment of the dam with copper sulphate to remove an algal bloom. Although the organism responsible for the bloom was not identified, cylindrospermopsin has been implicated in the episode (Bourke et al., 1983; Hawkins et al., 1985).

The present study was undertaken to determine appropriate conditions for effective removal of cylindrospermopsin by oxidation with chlorine. Preliminary experiments using low concentrations of purified cylindrospermopsin (≤ 30 μg l^{-1}) dissolved in ISO type 2 water, showed that chlorination can significantly decrease the concentration of cylindrospermopsin in water, where chloramination was not effective. Satisfactory degradation of the toxin in natural waters could be achieved

at pH 6–7.5 using a chlorine equivalent dose of 1–2 mg l^{-1} (Gray, 1996). The aim of this study was to determine the conditions which affect the degradation of cylindrospermopsin using chlorine and identify the conditions and chlorine concentrations required to achieve satisfactory degradation of cylindrospermopsin.

2. Materials and methods

2.1. Cyanobacterial material

A toxic *C. raciborskii* strain, AWT 205/1, developed and grown in our laboratory, was used for experimental work. The organism was grown in a 20 l Jaworskis medium (Thompson et al., 1988) continuous system. The culture is harvested as fresh Jaworskis medium is supplied to the culture vessel (peristaltic pump). This causes positive pressure within the culture vessel forcing culture material into a harvest bottle (2.85 l day^{-1}).

Cells were collected by passing the culture suspension through Whatman 541 filter paper. The cellular material was washed off the filter paper into aluminium trays and freeze dried. A quantity of freeze dried material (0.1 g) was dissolved in 500 ml of milli Q water, sonicated for 2 min using a Branson Sonicator (microtip, output control 5). The resulting dispersion was then centrifuged (5000 rpm for 15 min) and the supernatant was collected and filtered through 0.45 μm Durapor® membrane filters. The procedure produced a cell free extract stock solution with a concentration of approximately 400 μg cylindrospermopsin l^{-1}.

Purified cylindrospermopsin was prepared by methanol extraction of lyophilised *C. raciborskii* culture, and preparative HPLC (Chiswell et al., 1999). NMR and mass spectrometry confirmed the purity of this material to be 93.5% (Eaglesham et al., 1999).

Extracts were analyzed for cylindrospermopsin concentration by HPLC-tandem mass spectrometry (HPLC-MS/MS) (Eaglesham et al., 1999).

2.2. Sodium hypochlorite

Sodium hypochlorite was taken up with milli Q water giving a stock solution of 400 mg l^{-1} available chlorine.

2.3. Chlorine determination

At the end of the chlorination period, two 25 ml samples were removed from the reaction for free and total chlorine evaluation. Free and total chlorine were determined colormetrically by the DPD procedure (HACH, 1993) using a HACH DR/2000 spectrophotometer.

2.4. Total organic carbon determination

Total organic carbon was determined on 10 ml cell free extract samples, which were acidified to pH $\leq$ 2 with concentrated sulfuric acid upon collection. A Shimadzu TOC — 5000 total organic carbon analyser was used for the determination.

2.5. Protocol

Chlorination was performed in sealed Schott bottles containing 100 ml of a solution with a known concentration of cylindrospermopsin, either in a purified form or cell free extract. Following the addition of various sodium hypochlorite concentrations, bottles were resealed and occasionally stirred during the reaction period. At the end of the chlorination period 1 ml sub-samples were collected, immediately filtered into vials containing 25 μl acetic acid, to quench the chlorine reaction ready for HPLC-MS/MS analysis. These samples were analysed for residual cylindrospermopsin.

2.6. Effect of cylindrospermopsin concentration on the effectiveness of chlorine to degrade cylindrospermopsin

Experiments were carried out to determine the concentration of chlorine required for successful removal of cylindrospermopsin. Samples of cell free extract and purified cylindrospermopsin were diluted with milli Q water to give cylindrospermopsin concentrations between 15 and 185 μg l^{-1}. The effect of a range of chlorine doses (between 1 and 10 mg l^{-1} final concentration) was examined on each cylindrospermopsin concentration. Cell free extract was used to provide a source of organic matter (including geosmin and 2-methyl isoborneol) that is commonly present during a cyanobacterial bloom.

2.7. Effect of reaction time

Cell free extract solution (80 μg cylindrospermopsin l^{-1}) was exposed to chlorine, at chlorine concentrations of 2, 3 and 5 mg l^{-1}. Sub-samples were collected from the reaction mixture at time zero and at selected intervals during the incubation period. Reactions were undertaken in either an open (3 and 5 mg Cl l^{-1}) or closed vessel (2 and 5 mg Cl l^{-1}).

2.8. The influence of pH on cylindrospermopsin degradation

The effect of pH was studied using cell free extract (90 μg cylindrospermopsin l^{-1}), maintained at pH values between 4 and 9. The buffered extracts were subjected to chlorine doses of 3 and 5 mg l^{-1}.

Table 1
Results of chlorination study using pure cylindrospermopsin. The table presents initial cylindrospermopsin concentration before chlorination, total chlorine concentration after dosing, free chlorine concentration and final cylindrospermopsin concentration 30 min after chlorination started

Initial concentration of cylindrospermopsin (μg l^{-1})	Initial chlorine dose (mg l^{-1})	Concentration of free residual chlorine at end of 30 min reaction period (mg l^{-1})	Cylindrospermopsin concentration 30 min after chlorine added (μg l^{-1})
17	1	0.94	< 0.2
	2	1.9	< 0.2
50	1	0.95	< 0.2
	2	1.8	< 0.2
	3	2.9	< 0.2
90	1	0.90	< 0.2
	2	1.7	< 0.2
	3	2.7	< 0.2
	4	3.5	< 0.2
	5	4.5	< 0.2
140	1	0.90	< 0.2
	2	1.7	< 0.2
	3	2.7	< 0.2
	4	3.4	< 0.2
	5	4.1	< 0.2
185	1	0.83	0.81
	2	1.7	< 0.2
	3	2.7	< 0.2
	4	3.5	< 0.2
	5	4.0	< 0.2

3. Results and discussion

3.1. The effect of cylindrospermopsin concentration on the effectiveness of degradation using chlorine

The effect of various levels of chlorine on the purified cylindrospermopsin is shown in Table 1. In all cases, exposure of cylindrospermopsin to a chlorine dose greater than or equal to 2 mg l^{-1} over a 30 min incubation period resulted in reduction of the toxin level to below the detection limit (0.2 μg l^{-1}). Under these conditions, a chlorine dose of 1 mg l^{-1} was equally efficient in destruction of cylindrospermopsin at all except the highest toxin concentration. Reasons for this are unclear as the free residual chlorine level was sufficiently high. Table 1 also shows that in all cases, better that 80% of the original chlorine dose remains at the end of the reaction period. This is to be expected, given the purity of the toxin solution employed in these experiments.

A parallel set of experiments was also performed, using cell free extract (diluted to give cylindrospermopsin concentrations between 15 and 130 μg l^{-1}). The results

Table 2
Results of chlorination study using cylindrospermopsin administered as cell free extract. The table presents initial cylindrospermopsin concentration before chlorination, total chlorine concentration after dosing, free chlorine concentration and final cylindrospermopsin concentration 30 min after chlorination started

Initial concentration of cylindrospermopsin (μg l^{-1})	TOC (mg l^{-1})	Initial chlorine dose (mg l^{-1})	Concentration of free residual chlorine at end of 30 min reaction period (mg l^{-1})	Cylindrospermopsin concentration 30 min after chlorine added (μg l^{-1})
15	1.8	1	0.34	< 0.2
		2	1.1	< 0.2
35	2.4	1	0.20	3.6
		2	0.41	< 0.2
		3	0.92	< 0.2
60	3.9	1	0.27	38
		2	0.28	4.3
		3	0.33	1.3
		4	0.72	< 0.2
		5	1.2	< 0.2
100	4.0	1	0.19	91
		2	0.19	53
		3	0.15	6.8
		4	0.68	< 0.2
		5	1.2	< 0.2
		6	1.9	< 0.2
130	5.8	1	0.08	110
		2	0.10	58
		3	0.11	7.9
		4	0.34	< 0.2
		5	0.64	< 0.2

are shown in Table 2. As would be expected, increased chlorine doses were required in order to achieve satisfactory destruction of the toxin, particularly at higher levels of cell free extract. A minimal chlorine dose of 4 mg l^{-1} was required to reduce the toxin concentration below the detection limit with a cell free extract equivalent to 130 μg l^{-1} cylindrospermopsin. The increased chlorine demand as compared to the experiment with purified cylindrospermopsin is the result of additional organic matter in cell free extract. This is reflected in the lower concentrations of residual chlorine (about 10% of original dose) remaining at the end of the experiment.

3.2. Effective contact time for cylindrospermopsin degradation

Fig. 1(a)–(d) shows the effect of contact time, in both open and closed systems on the destruction of cylindrospermopsin by chlorine. In these experiments, the cylindrospermopsin was supplied as cell free extract, adjusted to give a toxin

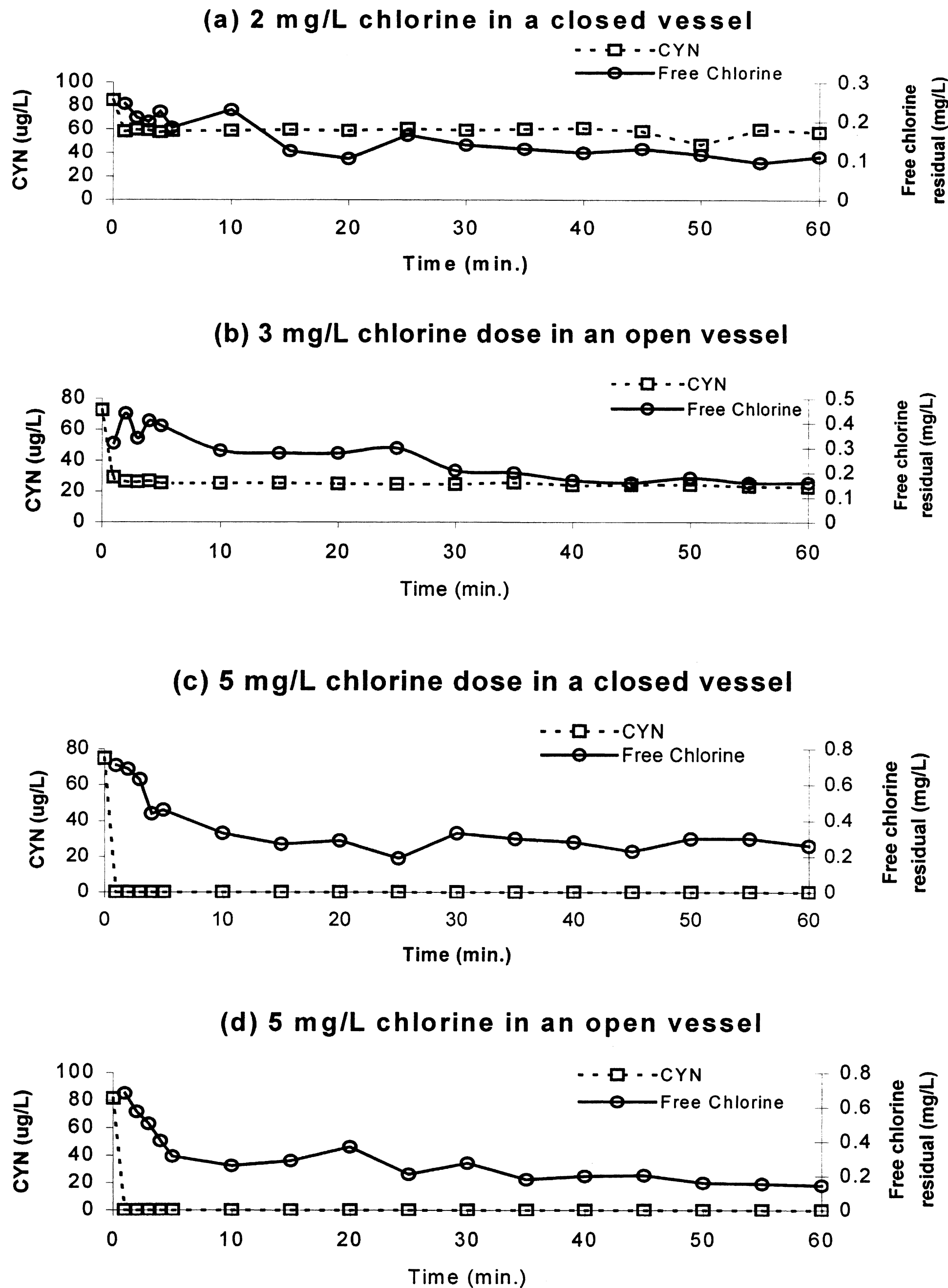

Fig. 1. Cylindrospermopsin (CYN) recovery following chlorination in open and closed vessels. Chlorine doses equivalent to 2 mg l^{-1} (a) and 3 mg l^{-1} (b) degraded 80 μg cylindrospermopsin l^{-1} by ~30%, within the first minute of contact. No further degradation was recorded over 60 min, noting that the free chlorine residual was <0.5 mg l^{-1} in these vessels. Increasing the chlorine dose to 5 mg l^{-1}, in both closed (c) and open (d) vessels, cylindrospermopsin was degraded to below detection limit (0.2 μg l^{-1}), within the first minute, here the free chlorine residual was >0.5 mg l^{-1} at minute one. All experiments are duplicated.

concentration of 80 μg l^{-1}. Chlorine doses of 2, 3 and 5 mg l^{-1} with a contact time of 60 min were employed. In all cases, toxin degradation was almost completely accomplished within the first minute with little further reduction in the level of cylindrospermopsin being accomplished over the next 59 min. The systems dosed with 2 mg l^{-1} (Fig. 1(a)) and 3 mg l^{-1} (Fig. 1(b)) chlorine, had 52 and 60% cylindrospermopsin reduction respectively within the first minute, with little reduction after that. Both the closed (Fig. 1(c)) and open (Fig. 1(d)) systems dosed with 5 mg l^{-1} chlorine, had $>99\%$ reduction within the first minute.

3.3. Influence of pH on cylindrospermopsin degradation

The influence of solution pH on the effectiveness of chlorination for cylindrospermopsin degradation (initial concentration 90 μg cylindrospermopsin l^{-1} cell free extract) was examined using chlorine doses equivalent to 3 and 5 mg l^{-1}. In Fig. 2 the percentage of cylindrospermopsin recovered after 30 min contact time is plotted against pH for the two chlorine doses used. A chlorine dose equivalent to 3 mg l^{-1} degraded cylindrospermopsin by more than 95% at a pH of 6 or greater. Only 60% of cylindrospermopsin was degraded when the solution pH was 5, and less than 15% of cylindrospermopsin was degraded when the pH was 4. However, while the cylindrospermopsin concentration was below the detection limit for pH values of 6 and more, using the dose equivalent of 5 mg l^{-1} of chlorine, low cylindrospermopsin concentrations were detectable at pH 5, and about 5% of cylindrospermopsin was detected when the treatment was performed at pH 4.

It has been suggested that the stronger oxidising agent hypochlorous acid is responsible for the degradation of the cyanobacterial toxin microcystins, at pH $<$ 8 (Nicholson et al., 1994). Our results suggest that cylindrospermopsin has reduced degradation at low pH, tending to imply the hypochlorite ion is important in the degradation of cylindrospermopsin. At pH $<$ 6, increasing the chlorine dose does lead to further cylindrospermopsin degradation, though residual toxin remains. At these pH values the free chlorine residual present is three times the threshold (Fig. 3). Hence the dissociation of the chlorine ions does not completely explain why cylindrospermopsin degradation is limited at low pH. The cylindrospermopsin molecule may be more stable at low pH. Similar results are true for PSP toxins, where there is limited degradation at low pH, with significant increases in chlorine doses required for degradation (NRCET and QHSS, 1997). The PSP molecule is more stable at low pH (Alfonso et al., 1995; Louzao et al., 1995).

Three mechanisms of cylindrospermopsin degradation exist: addition, substitution and oxidation. Addition of chlorine across the double bonds in cylindrospermopsin is expected to be a slow reaction occurring under weak oxidative conditions. Substitution and oxidation are more important reactions in water treatment and occur more rapidly at high pH rather than low (Johnson and Jensen, 1986). Our results follow that either oxidation or substitution is the

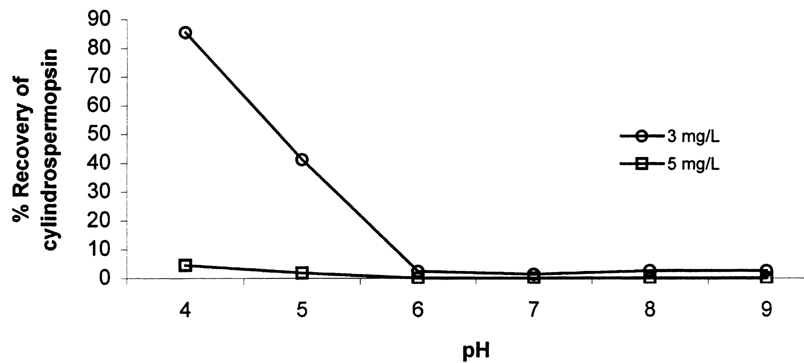

Fig. 2. Duplicated results for the percent recovery of cylindrospermopsin (CYN) after chlorination with equivalent doses of 3 and 5 mg l^{-1}, over a range of pH values. Degradation is greatest at pH ≥ 6. Increasing chlorine dose from 3 to 5 mg l^{-1} at pH ≤ 6 increases cylindrospermopsin degradation, though complete cylindrospermopsin degradation was recorded at low pH.

mechanism of cylindrospermopsin degradation, as toxin degradation is limited at low pH (Fig. 2).

4. Conclusion

As no guideline values have been set for cylindrospermopsin in drinking water, establishing methods for greater than 99% toxin removal are required. The study demonstrates that chlorination can be an effective method for cylindrospermopsin

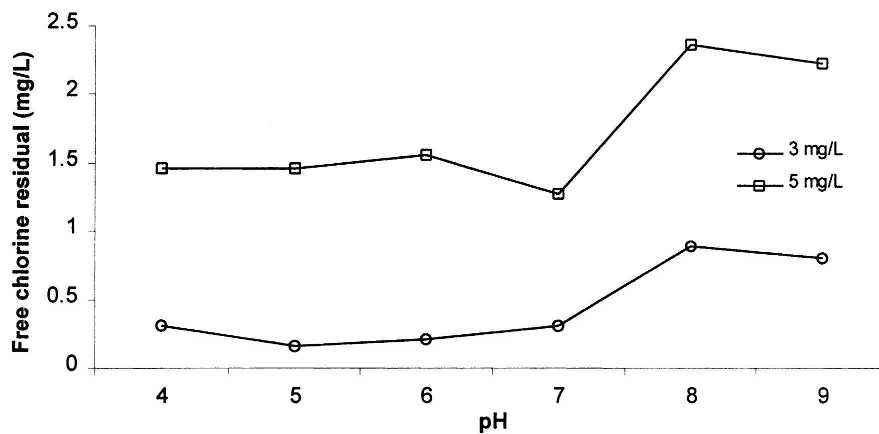

Fig. 3. Free chlorine residual present after 30 min contact time with cylindrospermopsin (CYN) (90 μg l^{-1}) for two chlorine doses 3 and 5 mg l^{-1}. Depicting the presence of a free chlorine residual >0.5 mg l^{-1} at all pH values at a dose of 5 mg l^{-1}. Free chlorine residuals tend to increase as pH > 6. All experiments are duplicated.

degradation in water treatment. The level of organic matter present in water influences chlorination efficiency substantially (cell free extract solution requiring higher chlorine doses than purified toxin sources). Hence the effectiveness of chlorination for cylindrospermopsin degradation would be best at the end of the water treatment. General water treatment procedures include the use of flocculation, sedimentation and filtration, minimising the formation of harmful by-products such as trihalomethanes during disinfection. A chlorine dose resulting in a free chlorine residual of 0.5 mg l^{-1} after contact within a pH range of 6.5 and 8.5, complies with the Australian Drinking Water Guidelines (1996). However, chlorine has been shown to produce potentially harmful by-products during water disinfection (IPCS, 1999). As a result further investigations are under way for the identification of chlorinated by-products after cyanobacterial toxin degradation by chlorination.

Acknowledgements

We would like to thank Queensland Health, Scientific Services for the use of their analytical equipment. We would also like to thank Bob Gray of Brisbane City Council for his comments and reference to work carried out in his laboratory. The work is kindly supported by an ARC SPIRT grant with significant contributions from the Brisbane City Council, Gold Coast City Council, South East Queensland Water Board, The University of Queensland and Queensland Health, Scientific Services.

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