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Toxicity of Cyanide, Iron-Cyanide Complexes, and a Blast Furnace Effluent to Larvae of the Doughboy Scallop, *Chlamys asperrimus*

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Toxicity of cyanide to various aquatic organisms, particularly freshwater species, has been previously reviewed (USEPA 1984). However, studies on the effect of cyanides to molluscs, organisms widely used for toxicity evaluations, are few. An early study on the oyster, *Crassostrea* sp., showed that its gill ciliary activity was suppressed in 130 ppb cyanide within 10 minutes, and was inhibited in 30 ppm cyanide after 3 hours (Usuki 1965). The arcid blood clam, *Scapharca inaequivalvis,* was found to be vulnerable to anoxia even in oxic conditions caused by blocking of the respiratory chain in the presence of cyanide (deZwaan *et al.* 1993). The LT50 value (time to 50% mortality) of the clams in oxygenated water spiked with 1 mM (26 ppm) CN^- was 8.2 to 11.2 days.

This paper discusses the toxicity of NaCN, $K_3Fe(CN)_6$, $K_4Fe(CN)_6$ and a blast furnace effluent on the embryonic development of the doughboy scallop, *Chlamys asperrimus.* The sublethal endpoint considered is abnormality in the development of the embryo into a shelled, straight-hinged larva during the first 48 hr. The purpose of this study, being part of a wider research, was to investigate the sensitivity to free and complexed cyanides of organisms typically found in Australian waters.

MATERIALS AND METHODS

Toxicity testing was performed in an 18°C-conditioned testing room. A Labec environmental cabinet, set at 18±1°C and 16 hr light : 8 hr dark cycle (<1000 lux, cool white fluorescent tubes) was used for incubating the test organisms during the experiments. Fifty-mL glass tubes (10-cm high, 2.5 cm in diameter, flat bottom) were used as test containers. All glassware was washed initially with detergent and tap water, soaked overnight in 10% HNO_3, rinsed with research-grade water, and air dried.

Seawater used in the bioassays was collected from Rose Bay, NSW and stored in 5 kL epoxy-lined concrete tanks until required. It was filtered through 10- and 5-µm membrane filters, UV-irradiated and adjusted to 18°C approximately 24 hr prior to use. Laboratory research grade water (Modulab Analytical) was used for the preparation of stock standards and reagents for analyses. All reagents used were analytical grade, unless otherwise indicated. Stock solutions of NaCN, $K_3Fe(CN)_6$, $K_4Fe(CN)_6$ and $CuSO_4$ were prepared fresh when required. Reagents required for chemical analyses of free and total cyanides were prepared weekly or daily.

Effluent evaluated for toxicity to larvae of *C. asperrimus* was collected from a blast-furnace operation of a metal-processing plant in South Australia. When the effluent

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(in a well-sealed polyethylene-lined steel drum) was delivered to the laboratory, it was transferred immediately to 10-L polyethylene containers and kept in a dark room at =<4°C until required for toxicity tests. Prior to the test, a representative sample, which included insoluble matter, was diluted 25-fold in seawater (the approximate dilution of the effluent prior to discharge to receiving waters). It was then filtered through Whatman no. 1 filter paper, with care taken to prevent its exposure to ultraviolet light and minimize losses of HCN. Salinity and pH were measured and adjusted, when necessary, to match those of the dilution seawater. This diluted and filtered sample served as 100% effluent. It was stored in a tightly-capped 1-L polyethylene bottle and allowed to acclimate at 18°C 24 hr before the bioassay.

Analytical methods used in the determination of cyanides were adopted or modified from standard methods for water and wastewaters (Clesceri *et al.* 1985). Stock NaCN solution (1000 ppm as CN) was standardized against $AgNO_3$ to the first permanent white precipitation endpoint. This NaCN solution served as the standard for the determination of free and total cyanides in all test solutions. The concentration of free cyanide was determined by direct measurement of the potential of a cyanide selective electrode (Orion model 94-06) against a saturated calomel reference electrode (Orion model 90-02) using an Orion model 250A digital voltmeter. Total cyanide was determined spectrophotometrically by the pyridine-barbituric acid method at a wavelength of 578 nm, following sample UV irradiation and distillation in phosphate buffer. The apparatus used was a Skalar Cyanide Segmental Flow Analyzer coupled with a Cecil CE 1010 spectrometer and Skalar ED 41 recorder.

Measurements of pH were done with a Microcomputer pH-VISION Model 6007, salinity using a YSI model 603 S-C-T meter, and relative % saturation of dissolved oxygen using a WTW OXI 92 dissolved O_2 meter.

The method for 48-hr larval abnormality test of *Chlamys asperrimus* was that of Krassoi *et al.* (1995). Each bioassay consisted of 7 replicates of a control and 5 to 6 concentrations of cyanide, inoculated with equal populations of *C. asperrimus* fertilized eggs. These test solutions were incubated under optimum conditions for normal development to the D-veliger (prodissoconch I) stage for 48 hr. After 48 hr, five sets were examined microscopically to determine the proportion of abnormal larvae to normal D-veliger larvae. Data were used to estimate 48-hr EC50, LOEC and NOEC values. The remaining 2 sets were used for analysis of free and total cyanides prior to and at the end of the test.

Brood stock of the marine doughboy scallop *C. asperrimus* were collected from a depth of 15-20 m approximately 1 km off Murrays Beach, Jervis Bay, NSW. The scallops were transported wrapped in moistened hessian, stored in containers maintained at temperatures <= 18°C with ice. They were then brought to, and held in subtidally suspended prawn crates at Goat Island, Sydney Harbour until required for bioassays which were carried out within five days of collection.

Scallops collected from the holding facility in Goat Island were spawned in the laboratory within two hours. They were examined visually for gonad condition, and given a rating from 1 to 6 according to a system suggested by O'Connor and Heasman (1995 *in preparation).* Only scallops with a rating of >=5 (i.e., having gonads of uniform color and texture, or full with eggs or sperms and turgid acini) were selected for spawning.

At least three sexual pairs of scallops were selected and induced to spawn. The females were induced to spawn by first injecting the distal portion of the gonad with ~0.1 mL of 400 ppm serotonin (5-hydroxytryptamine creatinine sulphate monohydrate, laboratory grade). Injected scallops were each placed in individual 1 -L beakers containing 800 mL seawater. When the first female had begun to spawn (after 30-40 min), the males were induced to spawn in a similar manner. This was

done because the males spawn faster (15-30 min) than the females, and the spermatozoa should be as fresh as possible when mixed with the eggs to ensure a high fertilization rate.

Egg suspensions were combined and passed gently through a 100-µm sieve to remove debris. The suspension was then filtered through a 45-µm nylon mesh to retain the eggs which were resuspended in fresh seawater. The concentration of eggs in the suspension was estimated in a Sedgwick-Rafter counting cell under a microscope. The spermatozoa suspensions were also combined and passed through a 100-µm sieve to remove debris. The density of the spermatozoan suspension was estimated in an improved Neubauer haemocytometer under a microscope. A calculated amount of the spermatozoa suspension was added to the egg suspension to achieve an egg: spermatozoa ratio of 1:100. The egg/spermatozoa suspension was allowed to stand for 30 min for fertilization to take place. The required volume of the egg/spermatozoa suspension to achieve a density of embryo in the test solutions of 30±5/mL was calculated.

Seven replicates each of a control and 5 to 6 concentrations of the test chemical were prepared. The test solutions, prepared in seawater in the glass test tubes, were made up to 3 mL less than the final volume of 30 mL (to allow for up to 3 mL of embryo suspension to be added). They were covered individually with plasticwrap to minimize losses of HCN, then acclimated in the environmental cabinet for at least 2 hr prior to inoculation with embryos. Each of the prepared test solutions was inoculated with the calculated amount of the *C. asperrimus* embryo suspension to give a density of 30±5/mL. The volume of solution was finally made up to 30 mL. They were immediately incubated in the environmental cabinet for 48 hr under optimum conditions for development of the embryo to the larval D-veliger stage (18±l°C and 16 hr light : 8 hr dark cycle). An extra control solution was prepared for determination of % fertilization at 4-15 hr post-fertilization.

Each of the solutions in one of the sets was analyzed for free and total cyanides to determine actual concentrations experienced by the larvae. The pH, salinity and %saturation of dissolved O_2 were measured prior to cyanide analysis to ensure that optimal water quality conditions for larval development were being maintained.

After 48 hr incubation, each of the test solutions in the five sets was treated with 0.25 mL of 4% formalin in saturated $NaHCO_3$. This served to stop further larval development and to preserve the larvae. The preserved solutions were allowed to stand for a few hours to allow the larvae to settle to the bottom of the tubes. The number of normal and abnormal D-veliger larvae were then estimated. Counting of normal and abnormal larvae was carried out by placing 1 mL of the lowermost portion of the preserved solution (after drawing off the top ~28.5 mL) in a Sedgwick-Rafter counting chamber for examination under a dissection microscope at 100x magnification. A normal D-veliger larva was characterized by a perfect D-shaped shell constituting a straight-edged hinge connected to two half-disc shaped valves (Krassoi *et al.* 1995*)*. An abnormal larva was one that had failed to develop from a zygote to a trochophore stage or had an imperfect shell shape. The proportion of abnormal larvae was calculated for each test solution.

The test solutions in the additional set of tubes, untreated with formalin (because formalin and cyanide react), were analyzed for free and total cyanides. The pH, salinity and %saturation of dissolved O_2 of the solutions were also measured.

Statistical estimation of LOEC (lowest observed effect concentration) and NOEC (no observed effect concentration) values were performed using the TOXSTAT 3.3 package (Gulley *et al.* 1991). The % larval abnormality data were transformed by arc-sine$^{0.5}$, tested for normality of distribution by the Shapiro-Wilks test and variance homogeneity by Bartlett's test, before analysis of variance and Dunnett's test to

estimate the LOEC and NOEC values. Toxicity was expressed as the increase in the proportion of abnormal larvae in the test solutions compared with those of controls. The 48-hr EC50 value was estimated statistically by the trimmed Spearman-Karber method (Hamilton *et al.* 1977, 1978) following adjustment of %larval abnormality to correct for the proportion of larval abnormality found in the controls (Krassoi *et al.* 1995*):* Percent Abnormalities = 100 (%AT-%AC)/(100-%AC), where %AT is % abnormal larvae in test treatment and %AC is % abnormal larvae in controls.

Preliminary range-finding (to determine concentration range to use in definitive tests) and definitive toxicity tests for NaCN, $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ were conducted following the procedure outlined above. In all tests, the concentrations of free and total cyanides were determined prior to and at the end of each test. A test involving exposure of *C. asperrimus* larvae to 5 concentrations of a reference toxicant suggested by Krassoi *et al. (1995)* was carried out concurrently with each toxicity test. The reference toxicant chosen was copper sulphate at concentrations of 0, 2, 3.3, 6.4, 12 and 20 ppb Cu^{2+}.

The blast furnace effluent was tested to assess its toxicity to the larvae of *C. asperrimus.* The same test procedure described above was used. Five replicates each of the following concentrations of the effluent were prepared in seawater: 100%, 30%, 10%, 3%, 1% and 0% (control). The free and total cyanide concentrations were measured prior to and at the end of the test.

RESULTS AND DISCUSSION

Results of the preliminary range-finding tests for NaCN, $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ to larvae of *C. asperrimus* showed that the following concentration ranges were appropriate to use in the definitive tests: 0.005 to 0.10, 0.005 to 0.405 and 0.010 to 2.56 ppm for NaCN, $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$, respectively. (All concentrations here are expressed as total cyanide, as weight of CN. It should be noted, however, that for NaCN, the free and total cyanide concentrations are equal, whereas for iron cyanide complexes, the concentration as free cyanide is much less than that of total cyanide.) Initial results from these tests indicated that the larvae of *C. asperrimus* were extremely sensitive to cyanides compared with other organisms such as fathead minnow, cladocerans and amphipods (USEPA, 1984).

In toxicity tests, the measured average physicochemical parameters of solutions during the tests were: temperature 18±1°C, pH 8.01±0.03, salinity 31.6±0.9 ‰ and dissolved O_2 80.5±11.1 % saturation. The values fulfilled the test requirements for optimal development of the embryos to the normal D-veliger stage in the first 48 hr (Krassoi *et al.* 1995).

Doughboy scallop eggs used in the tests had an average fertilization success of 89.7% (coefficient of variation = 1.2%), measured approximately 15 hr after fertilization at which time they had developed into normal ciliated trochophores. At the end of the tests, an average of 82.6% (coefficient of variation = 3.4%) of the embryos introduced into the control solutions developed into straight-hinged D-veliger larvae. This indicated that the gametes produced by the brood stock were of good quality and, therefore, the test results were considered acceptable (Krassoi *et al.* 1995). For the controls and test treatments, larvae which failed to develop to the straight-hinged D-shaped veliger were considered as abnormal. They were characterized by imperfectly-shaped shells, usually with tissues extruding from the shells.

Table 1 shows the measured free and total cyanide concentrations in the test solutions prior to and at the end of the tests. Before commencement of the tests, the measured amounts of free or total cyanides closely agreed with the nominal values for NaCN concentrations. The slight differences could be attributed to variability arising from the analytical method. Similarly, the measured total cyanide concentrations were in

Table 1. Measured concentrations of free and total cyanides in tests of NaCN, $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ to larvae of *Chlamys asperrimus.*

Nominal concentration	At 0 hr		At 48 hr	
	Free cyanide (ppb CN)	Total cyanide (ppb CN)	Free cyanide (ppb CN)	Total cyanide (ppb CN)
NaCN (ppb CN)				
5	6.2	a	5.6	a
10	12.6	a	8.4	a
20	21.6	a	17.2	a
40	43.9	a	29.4	a
80	78.5	a	69.1	a
100	103.7	a	86.4	a
$K_3Fe(CN)_6$ (ppb CN)				
5	<dl[b]	6.8	<dl	5.6
15	<dl	15.8	<dl	14.7
45	24.9	46.0	22.1	47.1
135	33.3	135.1	31.2	131.6
200	39.5	208.7	35.7	211.9
405	53.2	412.9	55.9	406.3
$K_4Fe(CN)_6$ (ppm CN)				
0.01	<dl	11.5	<dl	8.7
0.04	<dl	46.3	<dl	40.2
0.16	26.7	164.2	24.3	158.1
0.64	35.6	619.4	30.8	624.1
2.56	168.7	2.55×10^3	176.3	2.48×10^3

[a]total cyanide = free cyanide

[b]below detection limit ~10 ppb CN

agreement with the nominal concentrations of the iron-cyanide complexes. However, after 48 hr, free cyanide had apparently been lost from the NaCN test solutions, with rates of loss greater in the higher concentrations of cyanide. The average daily loss of free cyanide was 10%. At a pH of 8.0 and temperature of 18°C, the proportion of volatile HCN in solution was approximately 96%. This obviously accounted for the losses in free cyanide concentration. This indicates that the larvae in NaCN solutions were being exposed to gradually decreasing concentrations of free cyanide over the 48-hr test. As for the iron-cyanide complexes, the free and total cyanide concentrations appeared to have decreased to a lesser degree after 48 hr. The test conditions had allowed exposure of larvae in the iron cyanide complex solutions to fairly constant concentrations of free and total cyanides.

Figure 1(a) demonstrates the effect of NaCN, $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ (expressed as total cyanides) on the proportion of larval abnormality in *C. asperrimus* when exposed to varying cyanide concentrations for 48 hr. The dose-response plots are non-linear, and show that the variability in % abnormalities obtained for a test treatment was generally low. The order of toxicity based on total cyanides to the scallop larvae was NaCN > $K_3Fe(CN)_6$ > $K_4Fe(CN)_6$. On the other hand, data in Figure 1(b), showing response plots based on measured 48-hr average free cyanide concentrations, demonstrate that the toxicity of the iron-cyanide complexes correlates well with the toxicity of NaCN. This indicates that the toxicity of the complexes was due only to the free cyanide ($HCN + CN^-$) component.

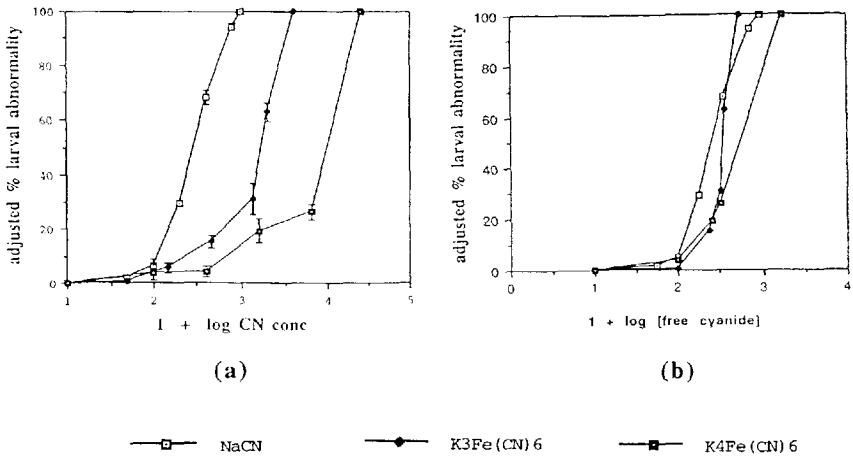

Figure 1. Toxicity of NaCN, $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ to larvae of *Chlamys asperrimus*, on the basis of (a) total cyanide and (b) measured 48-h free cyanide concentrations. (Bars, shown only in (a) but which apply also to (b), indicate standard deviations.)

Table 2 summarizes the estimated 48-hr EC50, LOEC and NOEC values obtained for NaCN, $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ to larvae of *C. asperrimus.* We believe that these are the first toxicity results obtained for NaCN and iron cyanide complexes to doughboy scallop larvae. The apparently low concentration of NaCN causing abnormality to the larvae was possibly due to the ease of absorption of HCN into the developing embryo. The crossing of HCN through the cell membrane is enhanced by the large surface area of the larva. Once inside the cell, HCN is readily converted to CN^- ion which may then inhibit normal activity of metalloenzymes (Eisler 1991). Comparison of the free cyanide acute toxicity value obtained here with those of other organisms reported in literature (USEPA 1984) shows that the larva of scallop *C. asperrirnus* is one of the most sensitive organisms studied so far. This is probably because a critical life stage in the development of the scallop was taken into consideration in the present study, i.e. the development of the embryo into a shelled D-veliger larva.

The 48-hr EC50 values obtained for the ferri- and ferro-cyanide complexes were both higher than the value for NaCN. However, examination of Table 1 and Figure 1 (b) shows that in the region of the estimated 48-hr EC50 values for the iron-cyanide complexes, the free cyanide concentrations were approximately 30 ppb. This concentration was very close to or within confidence limits of the 48-hr EC50 value

Table 2. Toxicity of NaCN, $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ to larvae of *Chlamys asperrimus.*

Test chemical	Estimated 48-hr EC50 (95% confidence limits) (ppb CN)	LOEC (ppb CN)	NOEC (ppb CN)
NaCN	28.6 (27.7-29.5)	10	5
$K_3Fe(CN)_6$	128 (123-134)	45	15
$K_4Fe(CN)_6$	686 (649-726)	160	40

determined for NaCN or free cyanide. This confirms that the toxicity was attributed to the free cyanide. Bound cyanides, $[Fe^{III}(CN)_6]^{3-}$ or $[Fe^{II}(CN)_6]^{4-}$ anions, contributed insignificantly to the toxicity of the complexes. This is in agreement with an earlier work on the effect of metal cyanide complexes to fish (Doudoroff *et al.* 1966). The lower concentration of $Fe^{III}(CN)_6^{3-}$ compared with that of $Fe^{II}(CN)_6^{4-}$ yielding an equivalent amount of free cyanide at the 48-hr EC50, indicates that the former is more dissociated in the test solutions. This is contrary to what would be predicted from the stability constants of ferri- and ferrocyanide which are 10^{42} and 10^{35}, respectively. Possible explanations for this are that the organisms preferably induced the dissociation of ferricyanide (over that of ferrocyanide) into Fe^{3+} and CN^- ions because they scavenged for ferric ions which is an essential trace element, or the test solutions may have been somewhat exposed to sunlight which caused decomposition of ferricyanide (USEPA 1984; Eisler 1991).

Reference toxicant tests using $CuSO_4$ to each batch of organisms were conducted concurrently with toxicity tests. The average 48-hr EC50, LOEC and NOEC values obtained were 7.2 (95% confidence limits: 7.0-7.4) , 3.3 and 2 ppb Cu, respectively. The LOEC and NOEC values were in agreement with those obtained by Krassoi *et al.* (1995), whereas the 48-hr EC50 value was similar to previous data obtained in the same laboratory (Lim 1995 *unpublished data).* The results indicate that the health of the scallop and test conditions were comparable with previous tests conducted in the same laboratory, and that the results of the cyanide toxicity tests were reliable and reproducible.

The blast furnace effluent examined was a complex mixture of waste products containing free and complexed cyanides and several other substances (Ian Hamilton *pers. comm.*). The measured pH and salinity of diluted effluent were 8.03 and 32.5 ‰, respectively, which matched those of the seawater used in the bioassay. Both absolute and adjusted % larval abnormalities in scallop larvae obtained from toxicity tests of different effluent concentrations are given in Table 3. At effluent concentrations of 3% and higher, proportions of larval abnormality were significantly higher than those in seawater controls based on Dunnett's test (α: =0.05). Estimated LOEC and NOEC values were 3 and 1% effluent, respectively, whereas the 48-hr EC50 value was 4.9%, with 95% confidence limits of 4.7 to 5.1%.

Table 4 gives the results of the analysis of free and total cyanides in each of the test treatments prior to and at the end of the bioassay. The table shows that both free and total cyanide concentrations decreased over the 48-hr test at a daily rate of 8.6 and 7.1%, respectively. Loss of volatile HCN during the 48-hr bioassay accounts for the decrease in concentrations of both cyanide species. Using the data given in Table 4, the corresponding free cyanide concentration for the 48-hr EC50 value could be interpolated from between the two closest effluent concentrations. Free cyanide

Table 3. Toxicity of a blast furnace effluent to larvae of *Chlamys asperrimus.*

Effluent concentration (%)	Mean[a] % larval abnormality	Mean[a] % larval abnormality (adjusted)[b]
0	15.1	0
1	17.0	2.5
3	44.2	34.3
10	77.9	73.9
30	100	100
100	100	100

[a]n=5 replicates

[b]Adjusted for larval abnormality in control

Table 4. Measured free and total cyanide concentrations in toxicity tests of blast furnace effluent to larvae of *Chlamys asperrimus.*

Concentration of effluent (%)	At 0 hr		At 48 hr	
	Free cyanide (ppb CN)	Total cyanide (ppb CN)	Free cyanide (ppb CN)	Total cyanide (ppb CN)
0	<dl[a]	<dl	<dl	<dl
1	8.9	19.5	7.8	17.2
3	17.1	32.4	14.7	28.2
10	48.5	92.7	41.2	79.7
30	130.4	253.4	106.9	215.4
100	429.3	832.9	340.1	691.3

[a]below detection limit ~2 ppb CN

concentration at the 48-hr EC50 was estimated to be 25.6 ppb (95% confidence limits: 24.7 -26.5 ppb). This value was slightly lower than the 48-hr EC50 value of 28.6 ppb obtained for NaCN alone (Table 2). However, considering the variability of the chemical analysis, the two values were not significantly different from each other. The amount of total cyanides in the effluent at the 48-hr EC50 did not correlate with toxicities of either of the iron-cyanide complexes. These results indicate that the most significant component of the blast furnace effluent that caused abnormality in larvae of *C. asperrimus* was free cyanide. The other components in the effluent that could also have contributed to the toxicity were metal ions and various complexed cyanides.

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