The half-life of cyanide in the blood of the marine fish, *Amphiprion clarkii* after cyanide exposure.

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ABSTRACT

Cyanide fishing continues to be used in the Indo-Pacific region to capture live reef fish for use by the live fish food and marine aquarium trades. The deliberate release of this broad-spectrum poison in reef environments is one of many anthropogenic threats to coral reefs today. Although this capture method is illegal in most countries, regulating its use is challenging due to the difficulty in determining whether a fish was captured using cyanide. A reliable method to test if marine fish have been caught using cyanide has long been the goal of the marine aquarium trade, but to date no test has been validated on fish with known cyanide (CN) exposure. Additionally, there are little to no toxicokinetic data on CN metabolism following CN exposure for marine fish. In this study, we exposed 38 specimens of *Amphiprion clarkii* to cyanide (50 ppm for 45 s) and measured the concentration of cyanide in their blood over time to characterize the detoxification process. CN was isolated from the blood by microdiffusion and derivatized to the highly fluorescent ß-isoindole and then quantified by HPLC. The half-life of cyanide in the blood of the marine fish *A. clarkii* was found to be 3.1 ± 2.0 hours. CN levels were seen to be slightly above control levels 1 day post exposure and below control levels 3 days post exposure suggesting that any test for CN exposure by measuring cyanide must be conducted soon after exposure. To our knowledge, this is the first direct measurement of cyanide in the blood of a marine fish following CN exposure.

KEYWORDS

Marine Aquarium Trade, Cyanide Fishing, Live Reef Food Fish, Microdiffusion, Toxicokinetics, Illegal Fishing

INTRODUCTION

Cyanide (CN) is a fast acting potentially deadly poison utilized by fishers in the coral triangle to capture fish from their reef habitat for the live reef food fish trade and marine aquarium trade (MAT) (Rubec 1986; Bruckner and Roberts 2008). A squirt bottle of concentrated cyanide solution (potassium or sodium) is sprayed by the fisher divers near the targeted fish paralyzing it for easier capture. All living things proximate to the squirt zone are also simultaneously exposed including corals and non-targeted fish species. The detrimental consequences of cyanide fishing on the reef and its inhabitants are well documented (Rubec 1986; Barber and Pratt 1997; Jones and Steven 1997; Cervino et al. 2003; Frey and Berkes 2014; Fabinyi 2016) and while the practice is illegal in most countries, it is difficult to regulate because of the lack of a robust test for CN in marine fish. Additionally little data are available on the toxicokinetics of CN metabolism in marine fish (Breen et al. 2019; Bonanno et al. 2021). Such data are needed to establish the time scale over which any test developed would be viable and would also establish the required sensitivity needed by any method employed (Losada and Bersuder 2017).

In the Philippines, the Bureau of Fisheries and Aquatic Resources' (BFAR) has used potentiometry after CN distillation (ASTM Method 500-CN-E) as a method to attempt to regulate illegal fishing activities (Manipula et al. 2001). This Cyanide Detection Test (CDT) was developed in the 1990's in collaboration with International Marinelife Alliance for testing fish homogenate and has recently been revisited to focus on liver and gill tissue homogenate because of the expected high blood flow through these organs (Cudia and Romero 2022). The method was reported to be viable to detect CN in spiked fish homogenate with a reported limit of quantitation (LOQ) of 0.039 mg/kg (Cudia and Romero 2022). However, when an attempt to use

the CDT on fish exposed to enough CN to induce mortality, CN was not detected in the post cyanide exposed fish (Mak et al. 2005). Mak et al. (2005) thus concluded that "the amount of cyanide necessary to kill fish is well below the amounts that can reliably be determined using the studied procedures." Hence while brief exposure to high CN concentrations can induce mortality, the fish do not absorb enough CN in a short, pulsed exposure to be detectable when the entire fish or organs are sampled as homogenate using the CDT method. This suggests that further improvements to the CDT must be made, perhaps focusing on ways to concentrate any CN recovered in the distillation procedure (i.e., minimize the volume of NaOH used in the absorber tube), or by testing the CN in the blood of marine fish directly as in mammalian studies (Bhandari et al. 2014).

The CDT was also used to study low dose CN exposure on freshwater Nile tilapia *Oreochromis niloticus* (Ramzy 2014). Ramzy (2014) reported exposing fish to a non-lethal dose of CN for 24 hours and the amount of CN in the gills, liver, muscle and blood was determined at 1 hour post exposure. The extended, sub-lethal exposure time allowed for the diffusion and accumulation of CN throughout the fish, resulting in a range of detectable CN levels. However, it's important to note that this is a single study, and further research is necessary to validate these findings.

In mammalian studies, rather than detecting CN directly, CN exposure is often inferred by the presence of elevated levels of the thiocyanate (SCN), the principal CN metabolite, in the blood or other bodily fluids (Logue et al. 2010). Absorbed CN is rapidly metabolized to SCN by the enzyme rhodanese in the presence of sulfur donors. In two recent studies elevated levels of SCN in *Amphiprion clarkii* and *A. ocellaris* following CN exposure have been reported confirming the

enzymatic pathway as a detoxification route in the marine fish as well as mammals (Breen et al. 2019; Bonanno et al. 2021).

A purported method of testing marine fish for CN exposure by detecting SCN in the holding water of fish exposed to CN using HPLC-OF or HPLC UV (Vaz et al. 2012). This paper has since been rebutted (Breen et al. 2018) and no other studies have replicated this work in the 12 years since it was first reported (Herz et al. 2016; Breen et al. 2018; Breen et al. 2019; Bonanno et al. 2021). The rebuttal by Breen et al. (2018) is based on using conservation of mass to calculate the dose of CN that the fish would have had to receive to excrete the SCN concentrations reported by Vaz et al. (2012). This calculated dose is ten times higher than the LD50 for CN in all living things (Breen et al. 2018). Vaz et al. (2012) also reported detecting elevated quantities of SCN in the holding water three weeks post exposure, even though the half-life for SCN elimination is on the order of days (Logue et al. 2010; Ramzy 2014; Breen et al. 2019; Bonanno et al. 2021).

Although many studies have reported on cyanide metabolism and toxicity in mammals (Logue et al. 2010; Jackson and Logue 2017), and two have reported on the CN metabolite SCN in marine fish (Breen et al. 2019; Bonanno et al. 2021), there have been no reports of the direct quantification of cyanide in marine fish exposed to cyanide. A preliminary study used CN radiotracers on the damselfish *Neopomacentrus violascens.* (Bellwood 1981). Fish were exposed to CN and then their organs were harvested. Inspection of the organs showed that CN is taken up across the gills and stomach and radiotracer analysis indicated that the CN was rapidly distributed throughout the high blood flow organs (Bellwood 1981).

Typically, blood is the most commonly used bodily fluid for determining cyanide exposure because cyanide has a high affinity to iron within globin proteins, and cyanide readily bonds to hemoglobin to form cyanohemoglobin (Jackson and Logue 2017). The formation of stable cyanohemoglobins disrupts the normal oxygen transport mechanism reducing oxygen availability to tissues leading to hypoxic conditions. Blood analysis for CN typically uses microdiffusion (Conway 1962) and derivatization (Lawrence and Frei 2000). Microdiffusion isolates the cyanide from the blood, increasing the specificity of the assay by removing the CN from the complex blood matrix (Roda et al. 2018; Okada and Miyaguchi 2020). Derivatization improves the sensitivity and the limit of detection of the measurement (Toida et al. 1984; Sano et al. 1992; Felscher and Wulfmeyer 1998; Tracqui et al. 2002; Ma and Dasgupta 2010; Roda et al. 2018; Okada and Miyaguchi 2020; Morikawa et al. 2022).

The goal of the work presented here was to detect CN in the blood of *Amphiprion clarkii* following CN exposure to establish the amount of CN in the blood and how fast it is eliminated as these data have never been reported for marine fish. To this end, we have measured the amount of CN in the blood of the marine fish *A. clarkii* for three days following a pulsed CN exposure. Free CN and CN bound to hemoglobin in the blood are isolated using microdiffusion and derivatized to the ß-isoindole fluorescent complex for analysis by HPLC. The blood concentration of the CN that we measure in the exposed fish is then used to compute the half-life for the elimination of CN from the blood. To our knowledge, this is the first report of the direct measurement of CN in the blood of a marine fish following pulsed exposure to CN.

METHODS

Materials

Sodium cyanide (NaCN), naphthalene-2,3-dicarboxaldehyde (NDA), taurine, and solvents were purchased from MilliporeSigma, St. Louis, MO, USA. Sodium hydroxide (NaOH) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). A stock solution of CN (10.0 ppm) was prepared in 10 mM NaOH and working standards were then prepared at various concentrations ranging from 0.1 ppb (µg/L) to 10.0 ppm (mg/L) using 10 mM NaOH as the diluent. A stock solution of NDA (4.0 mM) was prepared in HPLC-grade methanol and further diluted to 1.0 mM with phosphate buffer (pH = 8.0). A stock solution of taurine (5.0 mM) was prepared in phosphate buffer (pH = 8.0). Stock solutions were stored at 6°C when not in use and used within one week of preparation. Solutions used for capture and derivatization for both standards and blood samples were prepared just prior to use by mixing the stock solutions NDA, taurine, and 0.010 M NaOH 1:1:1 by volume, here after the CN capture solution.

Test Species and Seawater

All experiments carried out were approved by the Roger Williams University Institute of Animal Use and Care Committee (Approve #SR-21-06-08). *Amphiprion clarkii* of approximately 6-12 months of age were cultured at Proaquatix, Vero Beach, FL, USA, thereby ensuring no previous CN exposure. For all experiments, the temperature of the water holding the fish was maintained at 25°C by placing the holding tank containing the fish in a temperature-controlled room or water bath with a photoperiod of 14:10. Prior to exposure fish were fed pelletized food (Skretting Green Granule 1 mm) once per day. Fish were not fed following exposure. Water quality was maintained through daily water changes (100%) at a salinity of 30 psu with light aeration.

Natural seawater was used to house fish throughout the experiment. All seawater was produced using a standardized lab procedure. Briefly, seawater from Mt. Hope Bay, Rhode Island USA was pumped from 4 meters depth and mechanically filtered down to one micron, chlorinated in 8000 L storage tanks for 3-5 days. Before use, water was dechlorinated overnight with UV-sterilization, carbon filtration, and mechanically filtered to absolute one micron with a felt bag filter. Lab seawater was routinely buffered to an alkalinity of 2.2-2.5 meq/L with a final pH of 8.2 using sodium bicarbonate and sodium carbonate. Magnesium (1100 ppm) and calcium (380 ppm) levels are typical of 30 psu seawater.

CN Exposure

Thirty-eight live *A. clarkii* (3.0 ± 0.4 g) were pulse-exposed to a solution of 50 ppm CN for 45 s in seven groups. There were five groups of six fish, one group of five fish, and the final group contained three fish to account for mortality. For each exposure group, fish were transferred from a common holding tank to a basket. The basket with the fish was then immersed in the CN solution. After the 45 s exposure, the basket containing the fish was removed from the CN bath, and the fish were rinsed in two successive seawater baths. After the rinsing, the fish were housed in 7 polycarbonate tanks (Aquatic Habitats, 10 L tanks) containing static seawater with light aeration according to the exposure group.

Blood Collection

Four fish were taken haphazardly from the 7 exposure groups at the following time points: 9 minutes, 20 minutes, 40 minutes, 1.3 hours, 2.6 hours, 5.3 hours, 10.6 hours, 24 hours, and 72 hours post-exposure for blood collection. Five unexposed fish were used as controls. Fish were

then anesthetized with tricaine methanesulfonate (Western Chemical Inc., Ferndale, WA, USA) at a concentration of 200 ppm and buffered 2:1 with sodium bicarbonate in saltwater, dried, and weighed on an analytical balance. Anesthetized fish were prepared for blood collection by severance of the caudal peduncle using a #21 surgical blade. Upon severance of the caudal peduncle blood was collected in 40 mm heparinized microhematocrit tubes (Jorvet, Loveland, CO, USA) . Following blood collection, the fish were euthanized via pithing. The collected blood was combined into pre-weighed 125 µL heparinized microcapillary blood collection tubes (RAM Scientific, Nashville, TN, USA) and then re-weighed to determine the mass of blood collected.

Microdiffusion and Derivatization

CN from the blood was isolated via microdiffusion (Okada and Miyaguchi 2020). Five times the known blood volume of HPLC water was added to the blood sample, then vortexed and lightly centrifuged. The capture chamber consisted of a 20 mL scintillation vial equipped with TFE septa (Chemglass Life Sciences, Vineland, NJ, USA). The CN capture solution (100 µL) was added to a capture vessel (0.5 mL microcentrifuge tube) and then placed in the capture chamber. Finally, the diluted blood (100 µL) and 10% phosphoric acid (25 µL) was added to the capture chamber, taking extreme care to assure that the blood and the acid did not come into contact with each other until the lid was securely tightened on the capture chamber. Once sealed the vial was gently shaken to mix the blood with the acid and thereby initiate the capture process. The vials were then as specified by Okada and Miyaguchi (2020) for 2 hours at room temperature. Following microdiffusion and derivatization capture solutions were analyzed by HPLC with fluorescent detection.

Instrumentation

A Shimadzu i-Series Integrated LC-2050 with a Xbridge® BEH C18 column (2.5 mm, 2.1x50 mm) held at 40°C was used with an elution gradient. The gradient used was composed of water as mobile phase A and methanol as mobile phase B. Mobile phase B, initially at 25% was varied to 50% over the first 2.5 minutes, then returned to the initial conditions at 2.5 minutes where it was held for 2.5 minutes of re-equilibration. The fluorescent detector (Shimadzu RF-20A) used to analyze the NDA-CN complex was operated at an excitation wavelength of 418 nm and an emission wavelength of 460 nm.

Quantification

Calibration curves were made from derivatized CN standard solutions. Standards ranged from 0.1-50 ng CN in a volume of 50 µL. 25 microliters each of NDA and taurine were added, and the standards were left to react for 30 minutes at room temperature and then analyzed by HPLC. The calibration curve (9 points, r^2= 0.9987) was observed to be linear for CN quantities ranging from 0.1 - 50 ng. The mass of CN captured in the blood collected was determined by comparing the observed blood peak areas to those of CN standards. The mass of CN in the blood was then corrected for dilution and were thus reported as ng of CN captured per milliliter of blood collected, which resulted in a CN concentration in parts per billion. No correction was made for capture efficiencies which were on the order of 60-80%. The CN concentration was plotted as a function of time post-exposure and fit to a single exponential decay curve to determine the half-life (Breen et al. 2019).

RESULTS

Upon immersion in the 50 ppm CN bath, the fish quickly began to swim erratically and became completely immobilized in the exposure bath consistent with previous studies (Hanawa et al. 1998; Breen et al. 2018; Breen et al. 2019; Bonanno et al. 2021). There was one fatality in exposure groups 1 and 3.

Typical chromatograms shown in Figure 1 with the peak at 2.7 minutes corresponding to the derivatized CN product. The largest peak in Figure 1 corresponds to the CN level in the blood 10 minutes post exposure. An obvious loss of CN from the blood is observed when comparing the peak at 10 minutes post exposure to that of 10 hours post exposure, indicating the CN was rapidly eliminated from the blood of the exposed fish. A chromatogram for a control fish is also shown, and one sees that CN is still above control level at 10 hours. No peak corresponding to CN is observed in the control. Finally the chromatogram observed for the 25 ppb standard is shown indicating that at 10 minutes and 10 hours the CN levels are well above this value.

The averaged (n=4) concentration of CN in the blood as a function of time post-exposure on a log base 2 plot (Figure 2). The amount of CN in the blood was initially elevated and dropped around 5 -10 hours post-exposure. At 72 hours, the last sampling time, the CN in the blood was determined to be 47 ± 10 ppb, which was below the concentration observed in the controls (119 ± 50 ppb). Individual data points (unaveraged) were used to determine the half-life for CN elimination via fitting to single-phase exponential decay function which resulted in a half-life of 3.1 ± 2.0 hours ($r^2 = 0.38$ and $P<0.0001$), (see Table 1 for fit parameters).

DISCUSSION

The amount of CN in the blood of *A. clarkii* upon pulsed exposure to CN was measured as the fish metabolized the cyanide to thiocyanate. The early time data exhibited large standard deviations of the average CN concentration. This is likely due variable recoveries of CN in the capture solutions which is amplified by the higher CN concentrations found at earlier times (Okada and Miyaguchi 2020). Additionally, the expected natural variation found in biological samples will also contribute to the standard deviation (Parsons et al. 2009). Nevertheless, it is clear that the fish exposed to CN had elevated levels of CN in their blood, with the highest levels observed at the earlier times, and that after about 50 hours the model is within the average of the controls and at 72 hours is below the levels found in our controls (Figure 2). The half-life (3.1 ± 2.0 hours) determined is somewhat longer than the reported half-life in mammals (0.38-1.01 hours) (Logue et al. 2010) but this is not surprising as fish are ectotherms and thus metabolism is estimated to be about one-third of mammals (van de Pol et al. 2017). While the half-life is longer than that found in mammals, the elimination of CN observed is consistent with the initial increase of SCN in the blood plasma of the same species several hours post CN exposure (Bonanno et al. 2021). With this current study we have now determined CN under the same exposure conditions that we previously determined SCN (Bonanno et al. 2021) in *A. clarkii* and the CN and the SCN levels are compared (Figure 3). As CN is eliminated, it is converted to SCN and the modeled data show good agreement between the rapid metabolism of CN and the concomitant increase in the SCN which is also being eliminated from the blood (Breen et al. 2019; Bonanno et al. 2021) (Figure 3).

These data can be used to make a coarse estimate of the total cyanide absorbed by the fish, via the total blood volume. Fish in this study were on average 3.0 grams, a rough approximation would lead to such a fish having a blood volume of 0.18 mL if one assumes that fish blood volume is 6.0% of total mass (reported ranges are 5-7%) (Itazawa et al. 1983). The fit parameter A1 (Table 1) gives the concentration at time zero to be 543 ± 120 ng/mL, so therefore the fish absorb approximately 98 ng of CN or receive a dose of 33 ± 7 ng CN/g of fish or $0.061 \pm 0.01_3$ mg NaCN/kg. This compares well to the value reported for Nile Tilapia (0.118 ± 0.05 mg/kg) after receiving a sub-lethal dose for 24 hours (Ramzy 2014). Our value is lower than that in the tilapia study as it is for a 45 seconds pulsed exposure rather than a 24 hour exposure. Our value is likely lower than the actual total amount of cyanide absorbed by the fish as we have not corrected for capture efficiency (60-80%), as we have not accounted for any CN in gills or other organs and tissues. If total CN in an exposed fish is to be determined, it will be important to examine tissue and organs specific cyanide levels and compare those to blood levels. Additionally, the value could be larger because of the uncertainty in the fit parameters. Nevertheless, it is consistent with what one might expect for a sub-lethal dose in that it is lower than the LD50s reported in mammals (i.e. 6.44 mg/kg NaCN for a rat) which further corroborates that the CN toxicokinetics of marine fish and mammals have similar pathways and that marine fish have no capacity for supra-CN absorption. Indeed, our study had two fatalities suggesting that exposure to 50 ppm CN for 45 seconds is very near the lethal dose for fish of this size. In earlier studies mortality was higher for this concentration at 60 seconds for *A. ocellaris* and with fish of smaller mass (Breen et al. 2019; Madeira et al. 2020).

These results also suggest that the CDT, with its recently reported improved LOQ of 0.039 mg/kg (39 ppb) (Cudia and Romero 2022) is likely still not sensitive enough to detect CN caught fish by testing fish homogenate (whole fish or tissues), which is consistent with earlier findings (Mak et al. 2005). The CN concentration in the blood of *A. clarkii* under these exposure conditions was probably at most on the order of 1-2 ppm, but only immediately after exposure. In our exposure studies blood volume collected ranging from 10-30 μL for a 3 - 5-gram fish. Studies on larger fish, where larger volumes of blood could be drawn and tested as the fish detoxifies would likely be successful using the ASTM Method 500-CN-E method, if sufficient blood volume could be drawn, and care is taken to minimize the volume of NaOH in the absorber tube.

Marine fish follow the common CN to SCN biochemical pathway found in other taxonomic groups as they recover from pulsed CN exposure (Breen et al. 2019; Bonanno et al. 2021). Our suggests that marine fish are slower to eliminate cyanide from their blood than mammals but successfully eliminate it, metabolizing all cyanide absorbed less than 3 days after a simulated fishing event. The rapid metabolism of CN makes SCN a better candidate to screen for cyanide-caught marine fish if samples are collected more than 12 hours post-exposure. If CN or SCN is to be used as a test to establish a CN-free supply chains, the half-lives of blood and tissues as well as endogenous levels of additional species must be well described.

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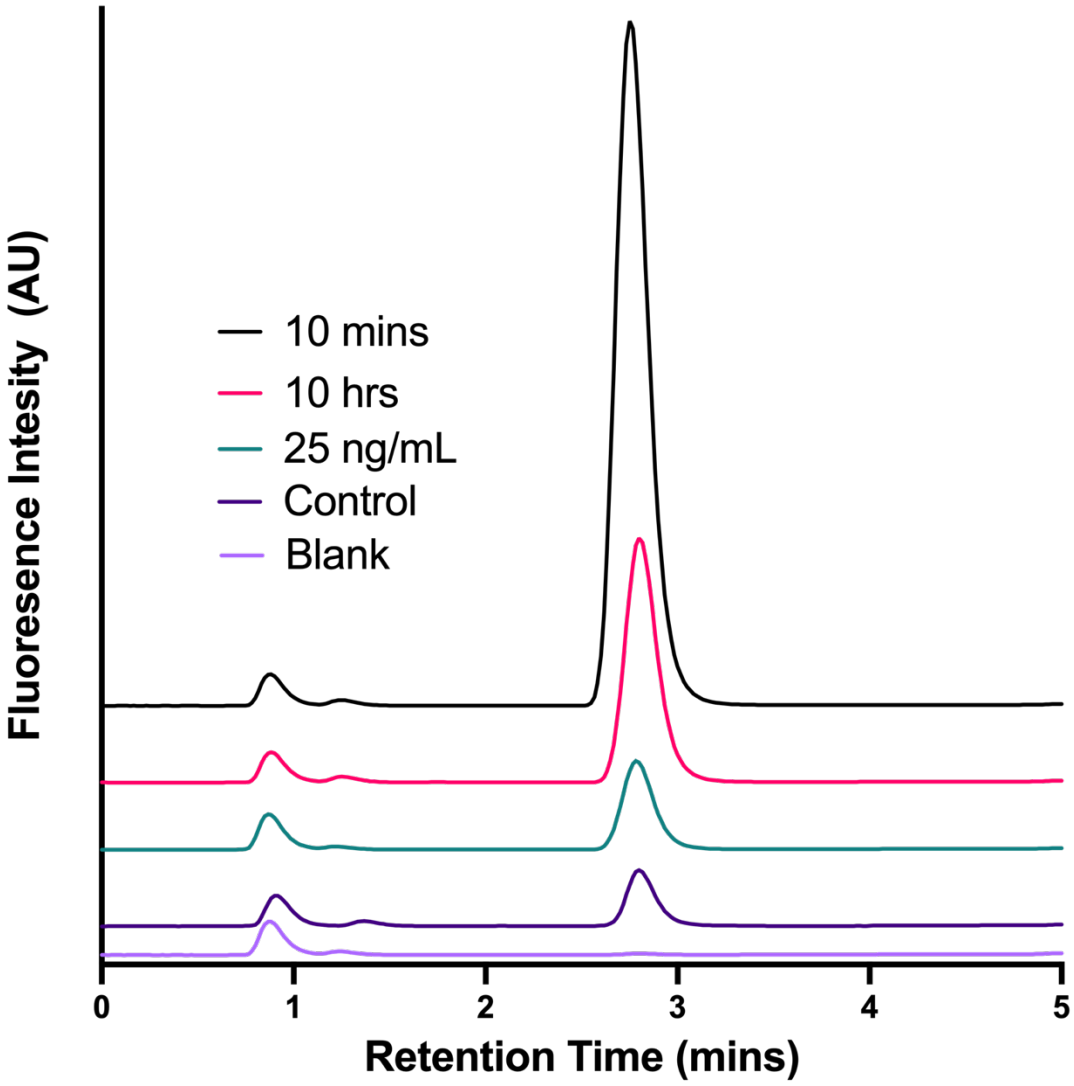

Figure 1. Typical HPLC chromatograms of CN derivatized to the ß-isoindole fluorescent complex. From top to bottom blood from fish 10 minutes post CN exposure, 10 hours post CN exposure, a non-capture standard spiked at 25 ng CN, a fish control sample, and a blank.

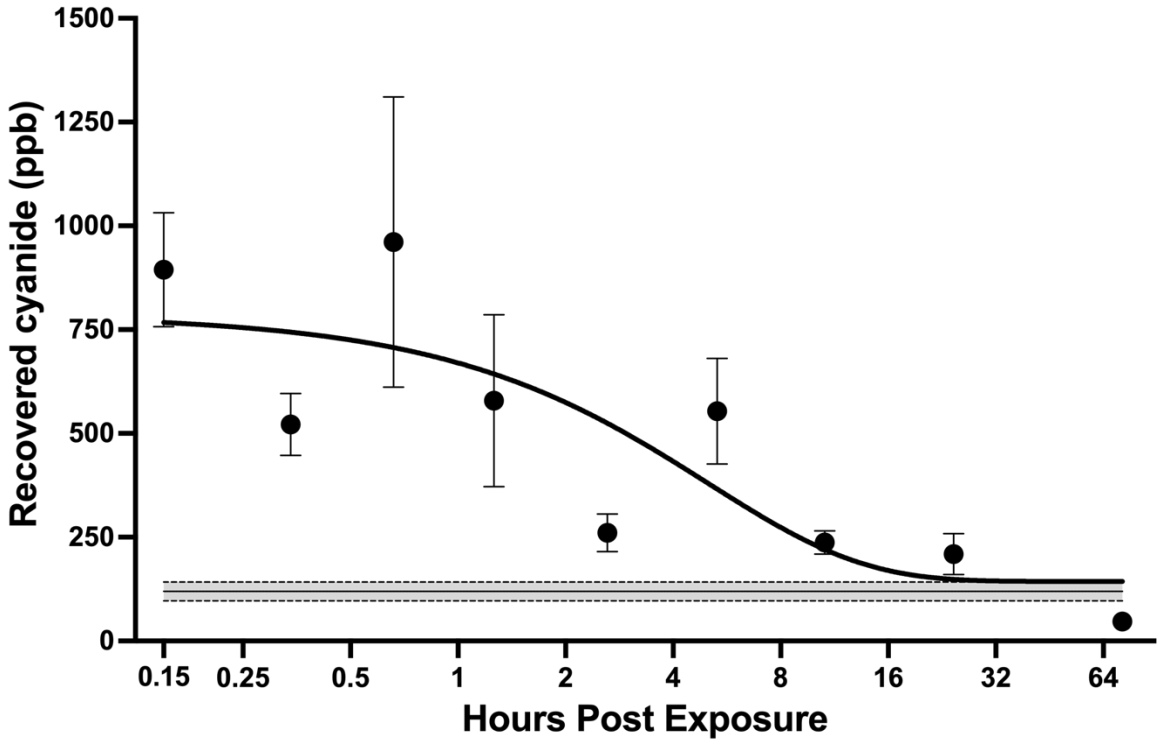

Figure 2. **Depuration curve for CN concentration in the blood of *Amphiprion clarkii* after exposure to 50 ppm cyanide (CN) for 45 s.** The solid line represents the fit of the data to a single-phase exponential decay function ($Y=A_1\exp(-x/t_1)+y_0$). Each data point (n=4) indicates the mean ± SE. The dashed line represents the average value observed in the controls. Where no error bar is observed, the error is smaller than the data point and equals four for each data point**.**

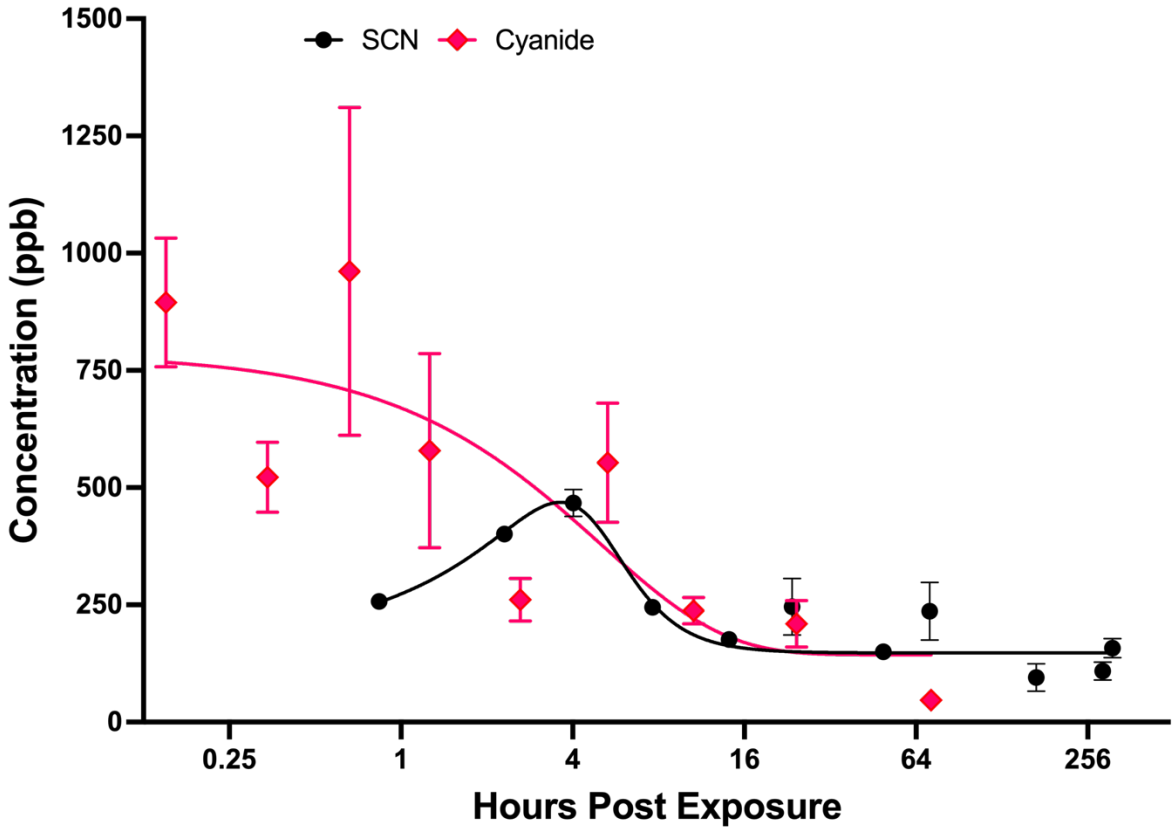

Figure 3. **Comparison of elimination curves for both CN (current data) and SCN (Bonanno et al. 2021) for *Amphiprion clarkii* after exposure to 50 ppm CN for 45 s**. Where no error bar is observed, the error is smaller than the data point. Cyanide (red line) fit of the data to a single-phase exponential decay function ($Y=A_1\exp(-x/t_1)+y_0$). Thiocyanate (black line) fit of data using the biphasic 5 parameter Brain-Cousens model.

Table 1. The results of the fit of concentration of CN in the blood versus time to a single exponential decay curve to determine the half-life for elimination of CN from the blood of *Amphiprion clarkii* upon pulsed CN exposure (45 s, 50 ppm). The functional form of the fit is $y=A_1\exp(-x/t_1) + Y_0$ where x is time post exposure. k and $t_{1/2}$ are calculated from the fit parameter t_1 via $k = 1/t_1$ and $t_{1/2} = 0.693/k$. The standard errors reported with the results of the fit are also given.

Parameter	
$t_{1/2}$ (hours)	3.1 ± 2.0
Y_0 (ng CN/ mL blood)	113 ± 93
A1 (ng CN/ mL blood)	543 ± 120
t_1 (hours)	4.4 ± 2.9
k ($hours^{-1}$)	0.23 ± 0.15
r^2	0.38
P	$P < 0.0001$