High-performance liquid chromatography to detect thiocyanate in reef fish caught with cyanide: A practical field application

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Introduction

The marine aquarium trade uses some of the most destructive fishing methods to coral reefs, which has evoked concerns for more than 30 years (Barber and Pratt 1998; Johannes and Riepen 1995; Jones 1997; Mak et al. 2005; Sadovy and Vincent 2002; Wabnitz et al. 2003). Yet, the worldwide trade in ornamental reef fish is flourishing. Import and export data suggest that marine ornamentals are worth USD 200–300 million annually, although complete trade statistics and current economic data are not available (Wabnitz et al. 2003; Larkin and Degner 2001).

Ornamental fish are often captured illegally using cyanide, an effective but very destructive fishing method (Barber and Pratt 1997; Johannes and Riepen 1995). The practice is meant to immobilize the fish, but if too much cyanide is applied, both targeted and non-targeted fish can die from overdoses (Cervino et al. 2003). The use of cyanide to capture fish is illegal in many Southeast Asian countries (Barber and Pratt 1998; Mak et al. 2005). In Indonesia, the use of cyanide has been illegal since 1985.[5] Between 1995 and 2005 the ornamental fish trade and its relationship to destructive fishing practices received much attention, both from scientists and the mass media. However, it is difficult to efficiently detect the presence of cyanide in captured fish, and in recent years there has been a reduced interest from the media and science, despite a flourishing trade.

There are several methods of detecting cyanide, using colorimetry, titrimetry or cyanide ion selective electrodes (ISEs) (Ikebukuro et al. 2013). Yet none of these techniques seem to be suitable for detecting cyanide in illegally caught ornamental fish because they are very time-intensive methods and require sacrificing the fish. Furthermore, the methods require a pre-treatment of the samples that involves acidification, heating and refluxing to evolve hydrogen cyanide (HCN) (Mak et al. 2004). The most prominent and promising cyanide detection test (CDT) until now was developed by the International Marinelife Alliance (IMA), Philippines and the Philippine Bureau of Fisheries and Aquatic Resources (BFAR) in 1992 (International Marinelife Alliance 2006). The test uses ISEs to detect concentrations of cyanide in homogenized organs of cyanide-captured reef fish (Bruckner and Roberts 2008). Although the half-life of cyanide is relatively short and it is quickly converted into thiocyanate (Mak et al. 2005), the CDT proved to be successful; 19% of 3,950 samples tested positive for cyanide (Barber and Pratt 1997). Despite the success, the testing method was abandoned in 2001 because like all other methods, the test required sacrificing the fish and was relatively time consuming (Mak et al. 2005; Graber and Siegel 2012).

Recently, a high-performance liquid chromatography (HPLC) technique was developed at the University of Aveiro in Portugal by Silva et al. (2011) to detect thiocyanate (SCN^-). Thiocyanate is a metabolite of HCN, converted by the enzyme rhodanese (thiosulfate sulfurtransferase; EC 2.8.1.1) and excreted by fish that are poisoned with cyanide (Isom et al. 2010). Another research group at the University of Aveiro was able to show that the modified HPLC technique can detect SCN^- levels of greater than 3.16 mg L^{-1} excreted by fish kept in artificial seawater (Vaz et al. 2012). Cyanide-poisoned clownfish (*Amphiprion clarkii*) were incubated in 1-L glass jars to depurate for 4 weeks. Water samples were collected on a daily basis and analyzed. Each day the fish were fed to satiation and the water from the jars was replaced by newly prepared seawater. Analysis of the collected water samples showed that each fish excreted a total of about 7.0 to 9.8 μgL^{-1} of SCN^- in the 28 days of depuration, on average. The newly developed method

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seems to be very successful in detecting low concentrations of thiocyanate. However, many questions remain unresolved. Under field conditions, when fish are captured by fishermen, factors such as cyanide concentration and duration of exposure are variable, and these factors might affect the excretion rates of fish. The depuration rate of thiocyanate can depend on the species and on the size and weight of the fish. It is known that various species of fish have different metabolisms and respiration rates, and the same is true for fish of different sizes (Jobling 1994). This leads to the conclusion that different species and sizes of fish incorporate different amounts of cyanide and have different cyanide conversion rates, thus leading to different SCN^- excretion rates. Additionally, parameters of natural seawater such as pH, salinity, levels of nutrients and traces of heavy metals (Gerdes 2001; Greenwood et al. 1997), as well as cyanide and thiocyanate (Silva et al. 2011), can vary on different reefs. The extent to which these factors limit the performance of the HPLC technique is not known.

The investigation reported on here aimed to extend the work of Vaz et al. (2012) and was designed to test a modified HPLC technique under field conditions, where cyanide exposure concentrations and exposure durations are unknown and natural seawater is used. Two distinct studies were conducted: Study 1 aimed to investigate the effects of varying field conditions on the thiocyanate excretion rates of five species of fish captured by fishermen. Study 2 was designed to compare thiocyanate excretion rates of two different fish species under controlled aquaria conditions and to investigate whether natural seawater influences the performance of the HPLC analyser.

Materials and methods

Study 1

Sampling was conducted in January 2013 in the region of Banyuwangi in east Java, Indonesia, in the facilities of a large-scale exporter. Cyanide is still widely used in the country due to a lack of effective law enforcement. Fish are brought to the facility on a daily basis by fishermen from the nearby villages and are held in tanks until packed into plastic bags for further transport to the main facilities in Bali and Jakarta. Fish species were selected for the study according to their capture rates and likelihood of being captured with cyanide. Cyanide is mainly used for solitary, fast-swimming fish and small fish that can hide in coral crevices (G. Reksodihardjo-Lilley, LINI, the Indonesian Nature Foundation, 19 December 2013, pers. comm., and a fisherman from Bali). Thus, the species *Pomacentrus vaiuli* (ocellate damselfish), *Centropyge bicolor* (bicolor angelfish), *Gobiodon quinquestrigatus* (five-lined coral goby) and *Amphiprion ocellaris* (clown anemonefish) were chosen.

The sampling procedure for thiocyanate detection followed that described by Vaz et al. (2012). Each fish to be tested for thiocyanate excretion was placed in its own aerated glass jar filled with filtered seawater and placed in a tank with a flow-through system to ensure stable water temperatures (Fig. 1). The fish were exposed to a natural day-and-night rhythm

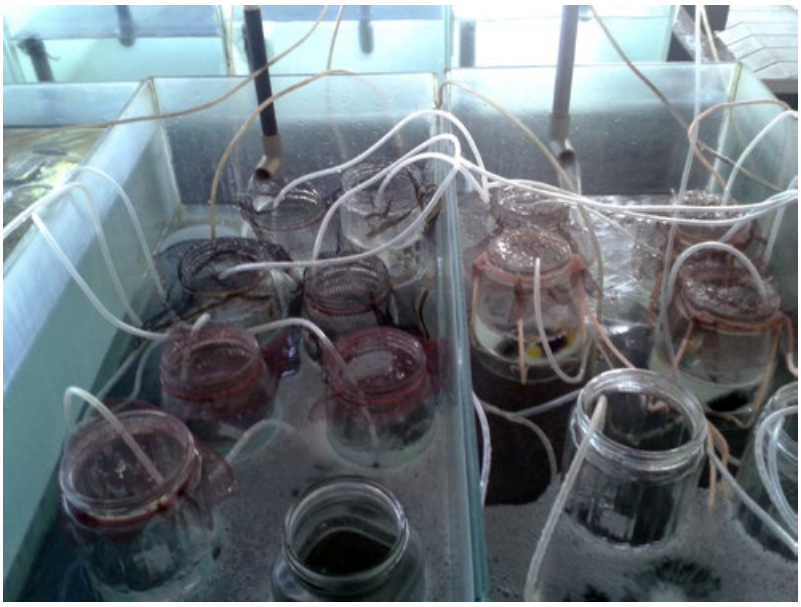

Figure 1. Experimental set up in Banyuwangi, Indonesia. All glass jars were placed in a narrow tank with a flow-through system and each jar was equipped with an aeration hose (photo by N. Herz).

of about 12.5 hours of sunlight in a semi-open hall. All fish were incubated for six days, which corresponded to one sampling round. Each day a water sample of 1.5 ml was taken and stored in a freezer at -20°C. Each day the fish were fed to satiation with "Sera Marin Granules" and *Artemia* nauplii (from Mackay Marine, USA) and the water was completely exchanged with fresh seawater. Due to the limited availability of fish, three sampling rounds of six days each were performed. In each round, three to four individuals per species were incubated; 8 ocellate damselfish, 8 bicolor angelfish, 11 five-lined coral gobies, and 10 clown anemonefish were tested for thiocyanate excretion (Fig. 2).

Study 2

The second experiment was performed at the MAREE aqualab of the Leibniz Center for Tropical Marine Ecology (ZMT) in Bremen, Germany. The aim was to compare thiocyanate excretion rates of two different fish species and to determine the effects of natural seawater on the performance of the HPLC technique. *Amphiprion frenatus* (tomato clownfish) served as a reference species to the experiment conducted by Vaz et al. (2012), and *Pterapogon kauderni* (Banggai cardinalfish) was included as a species from a different genus, because so far only fish from the genus *Amphiprion* had been tested. All fish used in this study were captive-bred in Germany to ensure that no fish had been exposed to cyanide. Two different treatments were applied to 10 individuals each from both species, plus a control with no cyanide application. In the treatments fish were exposed to a cyanide solution (KCN, 97% purity) of 12.5 mgL^{-1} for 60 seconds and to 50 mgL^{-1} for three times for 5 seconds, respectively (Fig. 3). The second treatment was intended to mimic the conditions under which fishermen stun and capture reef fish. After the treatments, all fish were placed in two consecutive cleaning baths and separately placed into 1.7-L glass jars for depuration (Figs. 4 and 5).

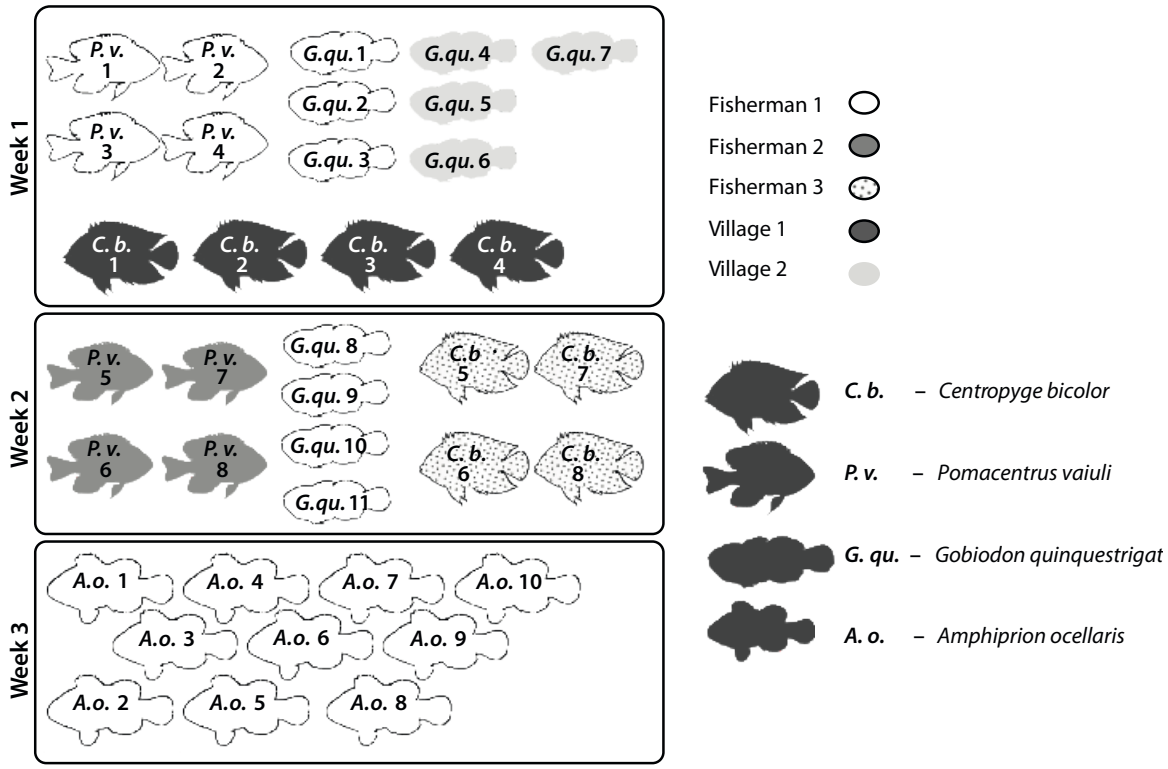

Figure 2. Over a period of six days, individuals of *Pomacentrus vaiuli* (P. v.), *Centropyge bicolor* (C. b.), *Gobiodon quinquestrigatus* (G. qu.) and *Amphiprion ocellaris* (A. o.) were incubated and water was sampled on a daily basis. Fish were captured by Fisherman 1, 2 or 3 from Village 1 or 2. During three sampling rounds (Week 1, 2 or 3) 8 individuals of each of the species P. v. and C. b., 11 individuals of G. qu., and 10 A. o. were sampled.

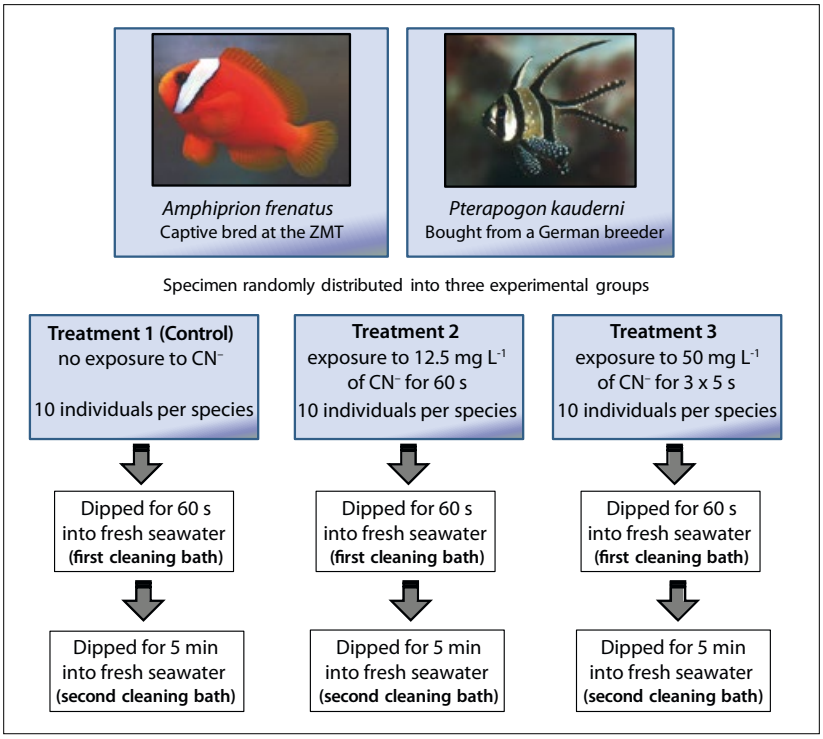

Figure 3.
Graphical illustration of the experimental design of Study 2. Individuals in Treatment 1 were not exposed to CN-; individuals in Treatment 2 were exposed to 12.5 mg L^{-1} of CN- for 60 s; and individuals in Treatment 3 were exposed to 50 mg L^{-1} of CN- for three times for 5 s.

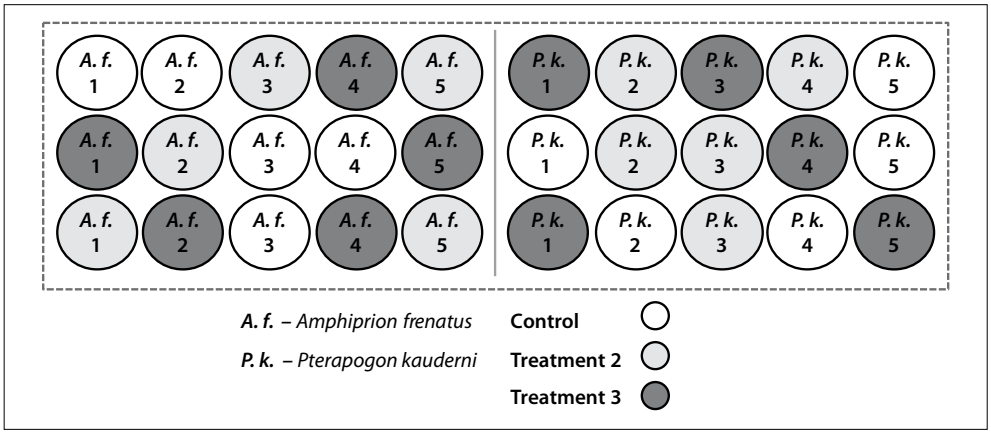

Figure 4. Illustration of the set up in the climate room of the Leibniz Center for Tropical Marine Ecology. All individuals from all three treatments were placed separately and randomly in 1.7-L glass jars, allowing them to depurate for two weeks.

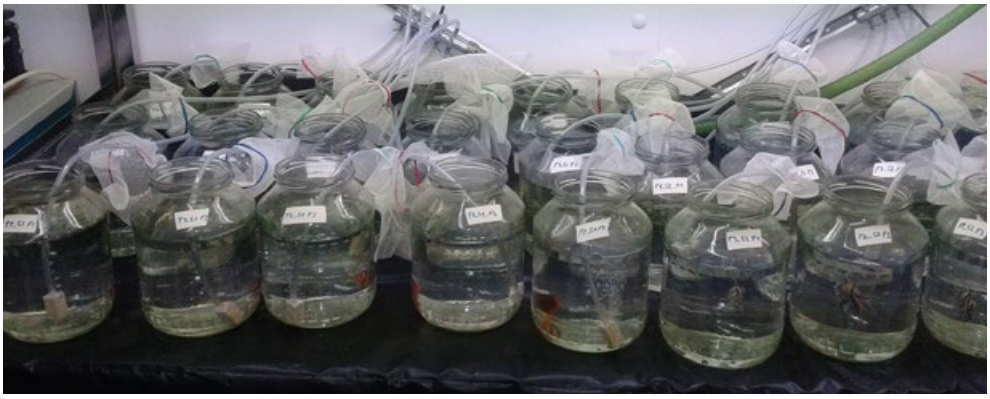

Figure 5. Experimental set up in the climate room of the Leibniz Center for Tropical Marine Ecology. In total, 60 glass jars with 30 individuals per species (10 fish per species and treatment) were incubated for 14 days. Each glass was equipped with an aeration hose. Water samples from the fish and a blank sample of seawater were taken at days 2, 5, 8, 11 and 14 (photo by N. Herz).

Following the cyanide exposure, all fish were randomly distributed among glass jars filled with 1,225 ml of natural filtered seawater from Wilhelmshaven. Each jar was equipped with an aeration hose for oxygen supply and placed in a climate room that was set at 26°C (± 1°C) (Fig. 5). Artificial light with a rhythm of 8:00–12:00 and 14:00–20:00 was used. Fish were kept for a period of 14 days. On a daily basis the fish were fed to satiation and the water was exchanged.

On days 2, 5, 8, 11 and 14, water samples from each glass jar and a blank water sample from the seawater tank were taken with a syringe and stored at -20°C.

At the end of the 14-day sampling period all fish were measured for length and weight (PLS 1200-3A scale by Kern & Sohn GmbH, Bahlingen, Germany). One-way ANOVA tests were performed to determine whether there were significant differences between the two species in terms of length or weight for each of the two experimental treatments. P-values of less than 0.05 were considered significant.

Water parameters

Parameters of the Wilhelmshaven water were tested twice, at the beginning and end of the incubation, using a colorimetric test for phosphate (PO_4) and nitrate (NO_3) and a DR/2010 spectrophotometer (Hach Company, Loveland, CO, USA). Temperature, salinity and pH were tested using a digital multimeter (Multi 3430 Set F by WTW GmbH, Weilheim, Germany).

Thiocyanate analysis

The water samples from both studies were analysed with an HPLC, including a Waters 2695 Separations Module and a Waters 2487 Dual Absorbance Detector (Waters Portugal, Lisbon, Portugal). The C30-column was slightly modified with 5% polyethylene glycol (PEG) and a more sensitive photodiode detector. The water samples were transferred to 1.5 ml glass vials and introduced into the injector unit. The column used a mobile phase of 300 mM sodium sulfate and 50 mM sodium chloride.

As described by Vaz et al. (2012), standard solutions of SCN^- (4, 50, 100, 200, 300 and 400 gL^{-1}) were used to calibrate a standard curve. The calculated detection limit lies at 3.16 mgL^{-1}; the retention time of thiocyanate is between 4.0 and 5.5 minutes.

Each sample was tested three times to calculate the standard deviation (SD) of the SCN^- peak. If the SCN^- peak of a sample turned out to be very small, the sample was spiked with a known amount of a thiocyanate solution and tested once more. The added amount of thiocyanate was then subtracted from the output value to calculate the actual amount of thiocyanate of the un-spiked sample.

Results

None of the water samples from the two studies could be analysed for thiocyanate. Instead, the HPLC analyser produced inconclusive chromatograms. In contrast to the samples from artificial seawater, which were spiked with thiocyanate (Fig. 6), no thiocyanate peak at minute 4.5 was detectable in the samples

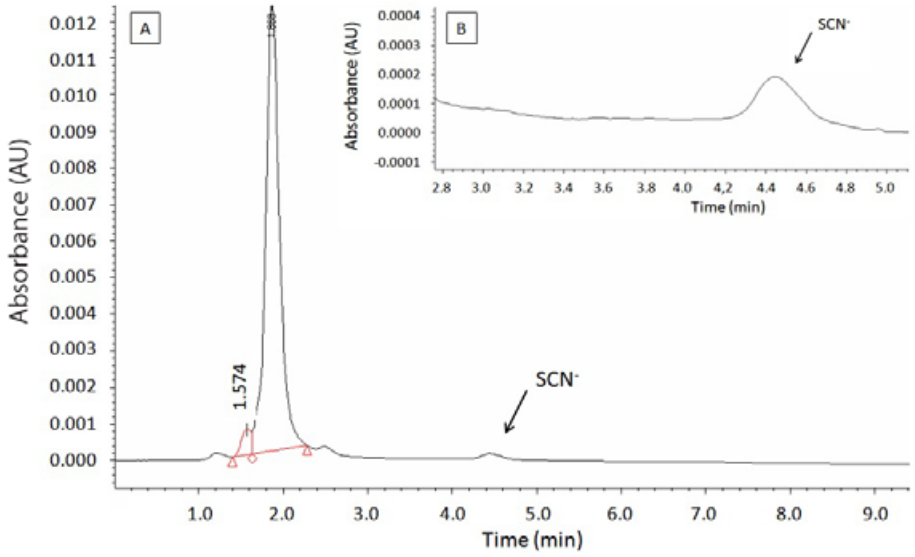

Figure 6. HPLC chromatogram of artificial seawater enriched with a certain amount of a thiocyanate solution (KSCN). Diagram (A) shows a chloride peak at minute 2.0 and a thiocyanate peak at minute 4.5; diagram (B) shows a higher resolution of the chromatogram and the SCN- peak.

from natural seawater, either in Study 1 or Study 2. All analysed samples resulted in chromatograms showing a huge peak of 0.822 absorbance units (AU) between minutes 6.5 and 9.0, which could not have been related to any substance (Fig. 7). Some of the samples from both Study 1 and Study 2 were spiked with a known amount of thiocyanate. Yet, no additional thiocyanate peak could be detected. Several tests were performed in order to determine factors that might have interfered with the SCN^- samples. A sample with a known thiocyanate concentration was spiked with an iron (III) chloride solution (Merck S.A., Algés, Portugal) to see if water contamination could influence the thiocyanate results (Fig. 8). Thiocyanate, being a pseudohalide, is able to form complexes with metals that might have prevented the detection of thiocyanate. However, the chromatogram revealed a clear thiocyanate peak at minute 4.5 (Fig. 8). The water parameters pH, salinity, PO_4 and NO_3 tested in Study 2 were all in the normal range.

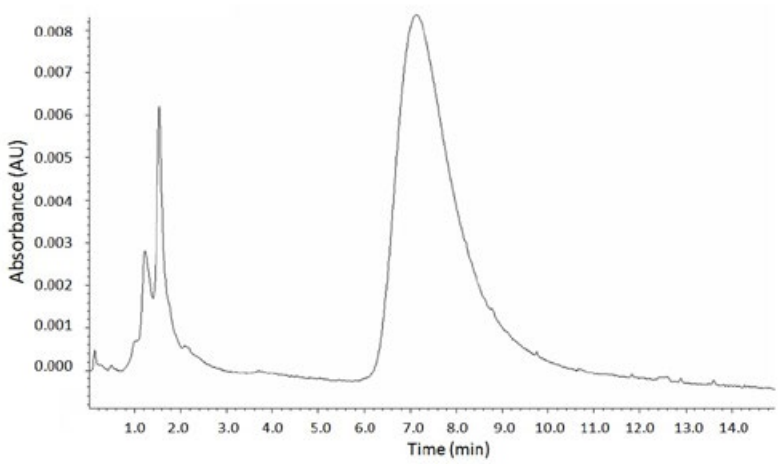

Figure 7. The absorbance profile of a sample, taken on 17 January 2013, generated by the HPLC. The sample contained water from day 6 of incubation of an *Amphiprion ocellaris* that was most likely captured with cyanide. It shows a biased chloride peak at minute 1.5 and an unknown peak at minutes 7.0–8.0. The expected SCN^- peak at minute 4.5 is missing.

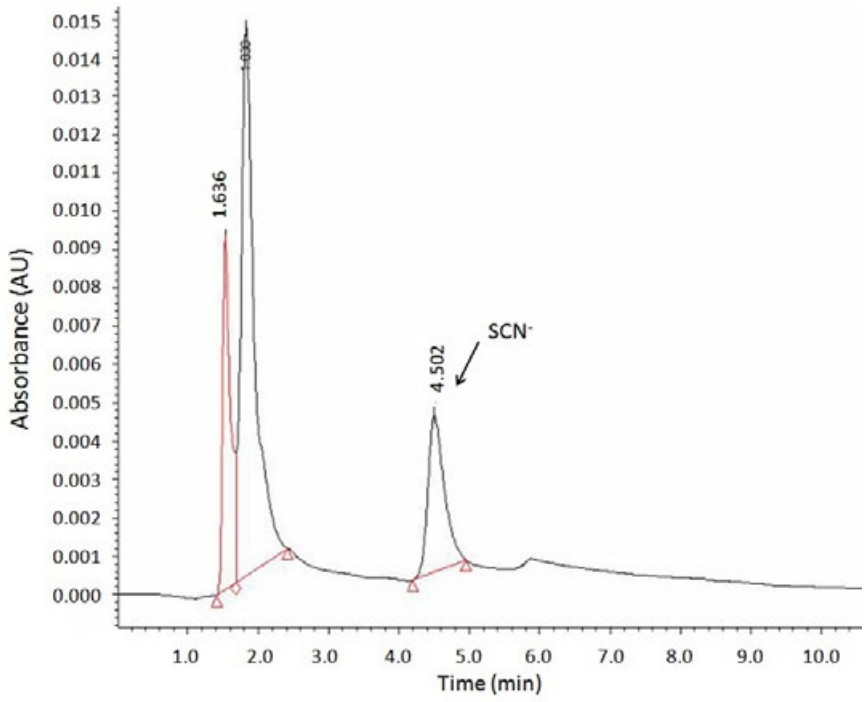

Figure 8. HPLC chromatograph showing the absorbance profile of an artificial seawater sample that was spiked with a known concentration of thiocyanate and ferric ions (Fe^{3+}). Thiocyanate still comes up as a peak at minute 4.5.

Discussion

Previous studies by Rong and Takeuchi (2004) and Silva et al. (2011) showed that the HPLC method can detect thiocyanate as an excretory byproduct of fish, provided that concentrations are higher than 3.16 mgL^{-1} and that artificial seawater is used. There may be reasons why the HPLC chromatographs of the analysed samples in this study did not show any thiocyanate. The main reason, however, might be that natural seawater is too complex with its many compounds and varying parameters to be suitable for the analysis of SCN^-.

Contaminations, unknown water contents or varying parameters might influence the performance of the HPLC, hindering the detection of SCN^-, as it might have formed complexes with transition metal ions, such as iron (III) (Gerdes 2001; Greenwood et al. 1997). Besides iron, SCN^-can also react with cobalt (Co(II)), copper (Cu(I)), gold (Au(I)), mercury (Hg(II)) or silver (Ag(I)) (Greenwood et al. 1997). SCN^- in such a bound form might not be detectable by the UV-Vis detector at l=220 nm or simply has an unknown retention time and unknown behaviour towards the C30-column (M.C.M. Vaz, University of Aveiro, 4 August 2013, pers. comm., and B. Meyer-Schlosser, University of Bremen, 11 December 2013, pers. comm.). Thiocyanate and iron (Fe^{3+}), for example, form thiocyanatoiron (III) ($FeSCN^{2+}$), which has a deep red colour, absorbing maximally at l=447 nm (Hovinen et al. 1999). In both experiments in this study the water that was used for the incubation came from the nearby harbour sites Banyuwangi and the Bay of Wilhelmshaven. Becuse both are harbour areas, pollution by ship ballast waters and agricultural and industrial sewage run-off is likely.

HPLC results also could have been influenced by the half-life of thiocyanate in seawater and its decomposition by bacteria. The half-life of thiocyanate in seawater is not known (V. Esteves and M.C.M. Vaz, University of Aveiro, 4 August 2013, pers. comm.). Plumlee et al. (1995, cited in Chaudhari and Kodam 2010) state that thiocyanate is a stable, non-hydrolysable, quite persistent compound. But it has yet to be determined what this implies for its detection by HPLC. It is known, however, that the amount of cyanide in a sample can change during storage, depending on the storage temperature (Calafat and Stanfill 2002; Lindsay et al. 2004; Lundquist et al. 1987), and the same could apply to thiocyanate. In the case of the samples from Study 1, problems in detecting the thiocyanate were expected because the samples were stored for a long time–from January to August, and they had to be transported for long distances several times. Samples from Study 2, however, were stored for only three months. The thiocyanate chromatograms of those samples should look different than those from Banyuwangi. Additionally, thiocyanate in seawater can be degraded by several species of bacteria, including Thiobacilli, Pseudomonads, Escherichia, Methylobacteria, Pseudomonas, *Klebsiella pneumoniae* and *Arthrobacter* spp. (Ahn et al. 2004; Chaudhari and Kodam 2010; Ebbs 2004). However, it is not known to what extent and at which rates microorganisms degrade thiocyanate in natural seawater. Studies to date have dealt with the artificial reduction of thiocyanate from mining wastewaters only (Akcil and Mudder 2003; Boucabeille et al. 1994; Chaudhari and Kodam 2010; Vu et al. 2013).

For Study 1 there are several other possible sources of inconclusive HPLC results. The amounts of cyanide used by fishermen might have been too small to detect, or six days of depuration might not have been sufficient. However, in Study 2 cyanide concentrations were the same or similar to the ones used by Vaz et al. (2012). Furthermore, fish were incubated for 14 days, which has been demonstrated to be a sufficient period for the excretion of thiocyanate.

Another factor possibly responsible for the absence of SCN^- peaks might be the HPLC method itself. The finding of such small thiocyanate peaks (Fig. 6) as were found in the studies by Silva et al. (2011) and Vaz et al. (2012) is not common. Their detection is difficult and the peaks could be part of background noise rather than true signals (K. Bischof and B. Schlosser-Meyer, University of Bremen, 12 November 2013, pers. comm.). Therefore, using the HPLC method to detect low concentrations of SCN^- requires further refinement. This study clearly revealed that it is not yet suitable for field use.

Conclusions

The results of the HPLC analysis made it clear that the method described by Vaz et al. (2012) is not yet suitable to detect illegally caught fish in the field or to prosecute poachers. Too many factors that might influence the results remain uncertain and the methodology is unable to detect thiocyanate in natural seawater.

Further investigation is also needed to determine the extent to which excretion of cyanide products varies by species, fish size, and method of fish capture. Apart from multiple factors of uncertainty, the HPLC method is quite expensive and requires a high level of expertise. Thus, it will be difficult for most institutions to acquire and maintain an HPLC instrument. For all of these reasons, it seems unlikely that a standardised HPLC methodology for thiocyanate detection can be developed in the near future. Other strategies and approaches to diminish cyanide use need to be considered.

Ethics statement

All experiments at the laboratory facilities of the Leibniz Center for Tropical Marine Ecology in Bremen were carried out in strict accordance with the German law (§ 8 Abs. 3 of the Animal Protection Law (TierSchG)) and permission to conduct them was granted by the ethics committee of the German health authority ("Senator für Gesundheit" in Bremen). The research in Indonesia was approved by the Indonesian Ministry for Research and Technology (Permit number: 4299/FRP/SM/IX/2012).

Acknowledgements

The authors wish to thank Dr Ricardo Calado, Dr Valdemar Esteves and Marcela M.C. Vaz from the University of Aveiro, Portugal, for analysing the samples and for providing insights into their HPLC methodology. Their help is highly appreciated. Additional thanks go to Dr Achim Meyer from the Leibniz Center for Tropical Marine Ecology for supporting the acquisition of the permit for animal experimentation and helping with the realization of the experiment at the center. Thanks go also to the managers and staff of the ornamental trading company in Indonesia for their cooperation, information, and for having us at their facilities to perform the experiments. Thanks are also extended to Gayatri Reksodihardjo-Lilley from LINI – The Indonesian Nature Foundation.

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