

Proceedings of the International Cyanide Detection Testing Workshop

February 6-8, 2008
Orlando, Florida

Edited by
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Front Cover Images: (Top) James Cervino – A fisher uses cyanide to catch marine ornamental fish; (Bottom) Stephen Why – Pens used in Micronesia to hold live reef food fish.

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Edited by Andrew W. Bruckner and Glynnis G. Roberts

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**NOAA Technical Memorandum NMFS-OPR-40
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I would also like to thank everyone for their cooperation and congenial interactions with other participants, given the complex and somewhat controversial nature of the problem. We had a very productive discussion of the issues surrounding the use of cyanide and options for

testing, as well as other holistic strategies to begin tackling this complex issue. I feel we made excellent progress in identifying possible next steps and look forward to working with all of you to implement the recommendations, and getting to the point where we can implement a network of CDT labs. I recognize the importance of the on-the-ground efforts geared toward communities dependent on coral reef resources, and commend all of the ongoing efforts in the Philippines, Indonesia and Vietnam. I am also happy to hear about the renewed interest of the government agencies in this issue, the progress that has been made, and the recognition of the importance of partnerships between exporting and importing countries to address this complex issue.

Andy Bruckner, May 1, 2008

PREFACE

The International Cyanide Detection Testing Workshop was conceived to identify possible options for reducing the use of cyanide in the capture of coral reef fishes for the marine aquarium trade and the live reef food fish trade. Because the emphasis was on identifying options for cyanide testing in exporting and importing countries, as well as management and enforcement opportunities, participants included forensic chemists with expertise in cyanide testing, as well as government and non-government representatives from the United States and three major exporting countries — Philippines, Indonesia, and Vietnam. Attempts were made to involve key experts who either had developed a possible test for cyanide or had implemented cyanide testing on marine fishes, as well as those involved in conservation and education initiatives directed at major stakeholders, including fishermen and other industry representatives and non-government conservation groups.

This workshop represents one component of a series of initiatives being implemented by the NOAA Coral Reef Conservation Program to address unsustainable and destructive trade in coral reef species. The mandates for this work include the Executive Order on Coral Reef Protection (13089) issued by President Clinton in 1998, the U.S. Coral Reef Task Force Coral Reef Action Strategy, and the Coral Reef Conservation Act of 2000. The Executive Order called for the creation of the U.S. Coral Reef Task Force (USCRTF), a multi-agency federal body chaired by NOAA and the Department of the Interior, with involvement by state and territorial governments. It also specifically identified unsustainable coral trade as an item the United States needed to address. Between 1998 and 2000, USCRTF members developed a road map for coral reef conservation — the National Action Plan to Conserve Reefs. One component of the Plan outlines seven key actions the United States should take to ensure the trade in coral reef species is sustainable, one of which targets efforts to improve law enforcement both domestically and internationally. At the 14th Meeting of the USCRTF in Palau, the Task Force called on its members to increase efforts to build enforcement capacity. The Steering Committee was charged with developing an enforcement “toolbox” in cooperation with the International Coral Reef Initiative (ICRI), to help coral reef management communities build enforcement capacity. A decision was also adopted by the USCRTF to address the use of poisons in the capture of reef fishes. The decision called for the creation of a working group on enforcement to 1) identify and recommend specific experts in law enforcement, field forensics, and toxicology/biomarkers; and 2) utilize their expertise to identify existing or potential cyanide detection methods or tests which could be used to determine if fish had been exposed to cyanide. They also asked the group to explore the usefulness of convening a broader expert panel to resolve the issues associated with cyanide detection tests.

Through funding from the NOAA Coral Reef Conservation Program, a workshop of experts was convened in February 2008. This document summarizes the outcomes of that meeting. Included are summary recommendations, working group reports, abstracts and white papers from speakers, and background information on cyanide fisheries. The Executive Summary highlights the major outcomes and conclusions from the workshop, including nine specific recommendations. These proceedings provide the framework for moving forward in implementing networks of cyanide detection laboratories.

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EXECUTIVE SUMMARY

The International Cyanide Detection Testing Workshop (February 6-8, 2008, in Orlando, Florida) brought together participants from Indonesia, the Philippines, the United States, and Vietnam, with representatives from fisheries and law enforcement agencies, forensic laboratories, CITES Parties, non-governmental organizations, industry, and academia. The primary objective of the workshop was to review the state of testing methods for cyanide and identify simple, cost-effective, rapid, and internationally accepted tests to detect cyanide or its metabolites in reef fishes at different points along the supply chain, including collection sites, export facilities, and ports of import. The workshop was organized by the United States (NOAA Coral Reef Conservation Program) with logistical support provided by SeaWeb, Kingfisher Foundation, and the International Marineline Alliance (IMA).

The workshop opened with a series of presentations on the extent of trade in live reef food fishes (LRFF) and marine aquarium fishes (MAF), patterns of cyanide use in these fisheries, and the impacts of cyanide on target and non-target species and coral reef ecosystems. A discussion followed on possible conservation, management, and enforcement strategies that have been implemented or are under consideration in the major exporting and importing countries to address the use of cyanide to capture reef fishes.

The participants noted that cyanide fishing is a widespread practice that has been reported in at least 15 countries or island territories. The pervasive use of this poison is driven by the lucrative growing and largely unregulated international trade in live reef food fish and the marine aquarium industry. The United States is the number-one consumer of MAF, while most LRFF are destined for Hong Kong and other Chinese markets. Species targeted by cyanide fishing include nearly all coral reef fish species, but its use appears predominant among the high-value MAF species such as surgeonfish, while LRFF trade frequently targets groupers and wrasses. Cyanide causes unacceptable levels of mortality of target species during collection and transport. Cyanide also causes mortality to non-target fishes and invertebrates including corals, and is associated with reef degradation as divers break apart coral to extract stunned fishes.

Potential methods for cyanide detection were reviewed during the workshop, focusing on their applicability to marine fish testing, including colorimetric methods, enzyme-based biosensors, cyanide-ion selective electrodes (ISE), and biomarker approaches. The International Marineline Alliance (IMA) created six CDT laboratories and tested over 48,000 specimens under contract to the Philippine Bureau of Fisheries and Aquatic Resources (BFAR) from 1993 to 2001. BFAR has continued to conduct cyanide testing using the ISE method on a more limited scale since 2001. Cyanide testing by the IMA for BFAR, in combination with other initiatives, led to a sharp reduction in fish testing positive for cyanide over the period 1996 to 1999.

However, at present only one Cyanide Detection Testing (CDT) laboratory remains operational full-time due to funding shortfalls and other issues, and cyanide use has subsequently increased. Testing done by the Puerto Princesa, Palawan laboratory found that 49% of the fish specimens

tested in 2004 had cyanide present (Rubec, personal comm.). Several alternative methods to detect cyanide in marine fishes have been published, but none of these have been applied to date as an enforcement tool. Conservation groups have also attempted to reestablish networks of CDT laboratories, with limited success.

The participants concluded that the most practical way to address cyanide use involves testing at points of export, and representatives from the three major exporting countries (Philippines, Indonesia, and Vietnam) supported implementation of networks of CDT laboratories in their countries. The working group also identified major limitations that need to be overcome for testing to be successful. First and foremost, recent studies have questioned the sensitivity of various tests, the ease of application by law enforcement in a field situation, and the potential difficulties in detecting cyanide at points of import due to the potential for rapid conversion of cyanide to thiocyanate and other metabolites. The participants suggested that the ISE method was the preferred test for use at points of export, mainly because it had been regularly used for many years. However, they also noted that sufficient concerns have been raised about the application of the ISE method to whole fish. For the ISE method to be internationally accepted as a standard test for marine fishes — especially in an enforcement context — the methodology used to test fish needs independent and careful verification.

After the conclusion of the plenary speeches, three concurrent working groups were convened to tackle questions surrounding cyanide testing and identify pragmatic approaches and collaborative initiatives in exporting and importing countries to mitigate the use of cyanide. The participants focused on the following topics:

- (1) existing or potential analytical cyanide detection methods that could be applied to marine fishes at collection sites, point of export, and ports of import;
- (2) requirements of an internationally recognized test at points of import that would carry weight in a court of law; and
- (3) steps, agreements, and/or partnerships that could be established between exporting governments, the U.S. government, NGOs, certification agencies, community groups, and academic scientists to implement cyanide testing in exporting countries.

The **Field Forensics and Toxicology Working Group** concluded serious gaps exist in the understanding of the metabolism of cyanide and its major metabolites in marine fish. A detailed analysis requires that the analyte is stable and at elevated levels in the fish long enough for it to be delivered to the laboratory and an examination conducted. Without a better understanding of these processes in representative fish species, the implementation of an exposure assessment method that would meet legal requirements would be difficult. The Working Group identified research as the highest immediate priority to determine the half-life of cyanide and major metabolites (especially thiocyanate) in marine fish and variations between species and life history stages. Other needs included an examination of background levels of cyanide (and major metabolites) in marine fish collected from different locations and whether they are above the limit of quantification, to ensure that the applied test can detect elevated levels of cyanide that would indicate exposure by fish collectors. To perform this research, validated methods for the determination of cyanide, thiocyanate and other metabolites (e.g.,

ATCA) in homogenized fish tissue must be used. These methods must demonstrate adequate sensitivity, specificity, accuracy, and precision.

The **Export Working Group** identified the need for a cohesive program in exporting countries to raise awareness among fishermen and other stakeholders, and improve capacity for training, testing, and enforcement. In particular, partnering with local communities, government agencies, and NGOs was recommended as the most practical way to address an issue of this magnitude and to increase technical capacity and secure financial support. Proactive plans must be made “on the ground” to engage the public, retrain and support cyanide-free fish collectors, and provide solid options for a cyanide-free network of exporters. An export testing scheme would require redefinition of export procedures and licensing to ensure testing compliance. Representatives from Indonesia, the Philippines, and Vietnam felt it might be useful for the three countries to meet to develop some common protocols for testing, enforcement, and education.

The **Import Working Group** suggested the need for harmonization of standards between import and export countries to eliminate policy gaps and tighten enforcement. Exporting parties should improve coordination and communication with importing countries, including sharing data on collection sites and fishermen, trade and fisheries, and exporters. Sharing such information could help identify legal and illegal shipments for importing countries, and potential collectors, middlemen, or exporters of concern. U.S. enforcement staff would benefit from knowing which companies repeatedly test cyanide-free, as well as those companies that tend to use cyanide. The group concluded there is an overall lack of comprehensive knowledge of each country’s requirements, resulting in confusion, corruption, and potential loopholes. Information on quarantine requirements, documentation needs, and the creation of a unified labeling and packaging system were all listed as possible ways to eliminate discrepancies. By establishing a tighter import policy and clear standards for compliance, the United States can provide stronger incentives for change in the industry.

SUMMARY RECOMMENDATIONS

During the final session, workshop participants discussed and formulated nine key recommendations necessary to mitigate the use of cyanide in the capture of coral reef fishes. The recommendations are presented here in priority order. There was general consensus that the primary issues to be resolved are gaps in our understanding of the cytokinetics of cyanide in marine fishes. The next critical steps involve validation of the existing ISE method used to detect cyanide at points of export, followed by implementation of this test at points of export. Further research on possible rapid field tests and tests to detect metabolites in fish tissue upon import should be undertaken. Upon confirmation of an internationally recognized and valid testing method for cyanide, networks of CDT labs should be established at major points of export and proficiency programs should be established for chemists.

Recommendation 1: Determine the pharmaco-kinetics of cyanide

Research is required to determine detectable levels of cyanide and cyanide metabolites in coral reef fishes and how these levels change over time following exposure. Without this knowledge,

it is difficult to identify the most appropriate cyanide testing methods that can be applied at different stages in the chain of custody, including collection sites, export holding facilities, and points of import, possibly 2 to 3 weeks after collection.

The concentration of cyanide present in the living fish will vary depending on the concentration of the cyanide solution used during exposure, the length of time of the exposure, time for holding and duration of transport, and post-collection treatment. Rates of metabolism may also vary depending on species or size. Research should encompass these possible variations such that it is possible to quantify measurable levels of cyanide in representative species and life history stages of marine fishes, and how these levels change over time, using at least two methods and multiple known concentrations.

To represent the time from initial collection and transport from the reef to holding facilities in exporting countries and then on to import destinations, fish samples should be exposed to varying concentrations of cyanide, with representative individuals sampled immediately after exposure and subsequent defined intervals. One aspect should also include a determination of detectable background levels, using fish from the wild caught with nets as well as captive-bred species. In addition, the effect of freezing samples must be determined for both field collection and delayed analysis samples to prevent misinterpretation of data obtained from frozen samples.

Recommendation 2: Validation of the Ion Selective Electrode (ISE) Cyanide Detection Test

Numerous methods are available to detect cyanide or cyanide metabolites, but most have been used only for water samples and blood; only limited testing has been done on whole fish and fish organs. Currently, the ISE method is the only cyanide test that has been applied on a large scale for MAF and LRFF at points of export, and testing has not been done substantially by enforcement agencies at points of import. The ISE method was successfully used in the Philippines from 1993 to 2001, with at least 48,000 fish tested; the test produced cyanide-positive results in fish up to 5 to 14 days after exposure (Rubec et al., 2003).

Representatives from Indonesia and the Philippines indicated that the ISE method is the preferred testing method, and both countries have secured necessary equipment, supplies, and trained staff to establish CDT laboratories. However, several studies have questioned the sensitivity of the test and the potential for false readings, and until these issues are resolved it is unlikely that importing countries will accept the results of testing using the ISE method. Although use of the ISE method for determination of cyanide concentrations has been validated through round-robin studies by the American Society of Testing and Materials (ASTM) and the American Public Health Association (APHA), these studies have not been performed on samples of digested fish, using the specific digestion method applied in the Philippines. IMA and affiliated laboratories have also developed supporting documentation to counter concerns expressed in the literature, but this has not been published in peer-reviewed journals or verified by an independent group.

Validation of the ISE method applied in the Philippines should be conducted by an independent laboratory not currently involved in marine fish testing to resolve questions about QA/QC, precision, and bias. This should include an examination of the sensitivity of the ISE method for fish samples, with emphasis on the distillation process and the recovery of cyanide from tissue samples. Results should be compared using at least one other recognized method. With some discretion exercised to limit the amount of testing, the ISE method can be validated in a relatively short time. The same cannot be said for any other method. A large amount of research will be necessary to develop methods to detect either thiocyanate or ATCA present in imported MAF, and to validate those methods.

Recommendation 3: Establish networks of CDT laboratories at points of export

A testing strategy based in export countries should focus on airport “choke points” in order to sample shipments before export. This approach would require fewer testing stations and would create better monitored and controlled testing conditions and procedures. This test must be rapid, credible, standard for all countries involved in the marine aquarium fish trade, and legally defensible, and it must utilize independently collected (e.g., blind) samples from shipments. It must also be able to accurately detect cyanide 5 to 14 days after the fish are exposed, as the time for transport to landing area and then landing area to export point frequently falls within this range. These networks should define and oversee their internal training and quality assurance programs and work with similar networks in other jurisdictions to establish and support an independent proficiency testing program.

Existing licensing schemes for exporting aquarium fish are often negligible, lax, and insufficient for impeding the use of poisons. Currently there are no official cyanide testing requirements in Indonesia, Vietnam, or the Philippines. It would be desirable to require that an export license and/or permit be linked to standards of compliance, incorporating poison-free capture and assent to regular cyanide detection testing. If possible, the export license should involve a suitable fee that would help underwrite the costs of testing and allow it to become self-financing (see Recommendation 6).

Recommendation 4: Obtain funding for establishment and maintenance of CDT networks in exporting countries

Funding sources should be explored to support the creation of CDT laboratory networks in the exporting countries (Philippines, Indonesia, Vietnam, and Malaysia) where cyanide fishing is most prevalent. Potential funding agencies include the World Bank, Asian Development Bank, or international NGOs.

Recommendation 5: Establish Training, Quality Assurance and Proficiency Programs for CDT labs and chemists

Training, quality assurance, and proficiency programs need to be implemented at cyanide testing laboratories. Many of the CDT procedures require extensive training and should be maintained to ensure performance consistency and accurate test results. A training program must be developed employing both didactic and hands-on training that develops a demonstrated acceptable level of performance, as defined by the performance characteristics of the specific test and the needs of the analysis. A quality assurance program should be based on ISO

17025 or similar structures and should at a minimum employ documented Standard Operating Procedures (SOPs), bench and blind QC samples, and statistically determined performance limits. A proficiency testing program should be conducted by an independent body and employ the use standard samples, unknown to the analyst, analyzed across the network and evaluated statistically to identify laboratory results that do not meet network performance standards. Frequency of testing should occur no less than “N” times per year.

Recommendation 6: Explore options for a simple field test at points of collection

Because of the considerable time and cost involved in quantification of concentrations of cyanide within fish, research is needed to identify and develop a quick, simple field test that could be used by enforcement officers at points of collection and at holding facilities in local communities. This test would only need to identify the presence of cyanide in fish immediately after capture, and not actual concentration.

The most feasible options currently include a low-cost colorimetric kit or ion selective electrodes (ISE) linked to portable ISE meters. Limited research of these options has been undertaken, but several limitations were noted—e.g., an inability to collect sufficient quantities of blood due to the small size of MAF and the need to digest fish tissue to liberate the cyanide, potential for interfering substances (e.g., sulfur), and other issues.

A protocol published by the American Society of Testing and Materials (ASTM D 5049), modified for use by the U.S. Food and Drug Administration (FDA) for the analyses of various foodstuffs including tuna, can successfully detect cyanide liberated from tissue digested in sulfuric acid. This simple test detects cyanide using Cyantesmo paper, which changes color from light green to blue in the presence of cyanide. This method may provide a rapid screening tool for marine fish samples at points of collection, allowing rapid analysis of multiple samples at low cost.

While a rapid screening test may be a deterrent, samples that test positive could be sent on to a larger testing facility where a more detailed test would be performed to quantify actual concentrations and to prosecute violations.

Recommendation 7: Testing and Accreditation Plan for Cyanide Free Fish in Exporting Countries

Exporting countries need a sound, government-instituted export licensing and certification scheme tied to cyanide testing at points of export. Similar to an accreditation approach, issuance of the license would be tied to confirmed cyanide-free status from a testing facility.

The group thought it would be feasible to implement some form of certification program if a suitable fee for the license were required. This would create a fund for the testing procedure and help make the system financially self-sustaining. In conjunction with export permitting, this method provides an incentive to reform any collectors currently using cyanide. Further, a database should be designed to track cyanide testing results; over time this information would illuminate patterns of use, mitigation success, and repeat offenders. License renewal could be linked to the testing program, with repeat offenders being unable to renew their license.

Importing countries should also require testing and verification by the exporting country (through certificates or other means) that the imported fish are cyanide-free.

Recommendation 8: Implement Complementary Legislation against Cyanide Fishing in Importing Countries

The officials of exporting countries at the workshop strongly advocate U.S. legislation regulating the ornamentals trade. This legislation would be fundamental for building political will and designing regulation and enforcement in exporting countries. The legislation or Code of Federal Regulations (CFR) could make U.S. imports of fish containing cyanide illegal, as well as clearly state standards for permissible imports (e.g., exporting partners must have testing system in place, species restrictions, habitat impact assessments and protection, etc.).

The Lacey Act makes it illegal to import, export, transport, sell, receive, acquire, or purchase fish, wildlife, or plants taken, possessed, transported, or sold in violation of a federal law, treaty, regulation, or Indian tribal law. It also is illegal for a person to import, export, transport, sell, receive, acquire, or purchase in interstate or foreign commerce: fish or wildlife taken, possessed, transported, or sold in violation of a state law, state regulation, or foreign law (16 U.S.C. §§ 3371-3378). Since most exporting countries have laws banning the use of cyanide and other poisons for fishing, a large portion of the aquarium fish imported to the United States (an estimated 90%) are illegal under the Lacey Act. However, enforcement of the Lacey Act is difficult and time-consuming. For example,

- A successful case must show importer knowledge and intent to import illegal fish.
- Origin countries must have good faith enforcement efforts in place.
- A U.S.-based investigation under the Lacey Act must be supported by the nation whose law or regulation was violated.
- In the case of cyanide, the fish samples would not be analyzed in the United States until three weeks after time of import because of backlogs and resource constraints. Shipments would have been processed, shipped to retailers, and sold by that point.
- The United States is unlikely to rely on accuracy of a cyanide test performed in exporting country.
- The standards for a test to be considered legally enforceable in the United States are high: the test would have to be peer reviewed, reliable, and in use for a period of time.
- Each case takes hundreds of hours per shipment to prove. An effective enforcement effort would immediately overwhelm the current system resources.

Federal legislation may be necessary to support the development and implementation of testing methods for thiocyanate and/or ATCA metabolites at the point of import, and to authorize funding in support of cyanide testing. A draft bill proposed by Representative Ed Case (Coral Reef Conservation and Protection Act of 2004 HR-4928) would have required some type of certification demonstrating that MAF imported to the United States were collected by sustainable means without the use of poisons such as cyanide. New U.S. legislation could require certification of fishes tied to cyanide testing by countries exporting MAF to the United States, but it may also require more comprehensive legislation in exporting countries and a

formal mechanism for evaluation and demonstration by the responsible party that the fish are in fact cyanide-free.

Recommendation 9: Develop a cyanide testing program in the United States

Participants felt that developing a cyanide testing program for points of import was a necessary step for deterring cyanide use. Unfortunately, such a system also presents the greatest challenge.

In one year there are over 11,000 shipments of non-CITES listed reef fishes into the United States. Currently, U.S. Fish and Wildlife Service (USFWS) import inspections focus on endangered and threatened species listed under ESA and CITES. Inspection of aquarium fish is a relative low priority, as none of the species of fish are considered endangered. In addition to a limited capacity to inspect each shipment, verification of cyanide is problematic because fishes may be imported several weeks after being caught with cyanide, which is known to rapidly break down into sodium thiocyanate and other metabolites.

Given testing backlogs for USFWS, samples would likely wait an additional three weeks after collection on import before testing. At this time, testing for cyanide metabolites is possible but extremely costly and time-consuming. In addition, there are limitations on the amount of time a shipment of fishes can be detained, as these the fish will die if not transferred to aquaria fairly quickly after arrival in the United States. Therefore, the challenge for the United States is multi-fold, and can be conflicting:

- Creation of a comprehensive cyanide detection testing program in the United States must address new, supporting legislation as well as increased funding and staffing needs.
- The U.S. government agencies involved with import law enforcement would need a workable, import point CDT test to confirm that fishes declared as being cyanide-free were in fact not contaminated with cyanide.
- Without this U.S.-based test, it would not be possible to enforce the Lacey Act or any new legislation created to address the use of cyanide.
- It would be ideal to supplement a U.S. import CDT scheme with cyanide-free certification from exporting countries. Although certification alone would not be enough for U.S. standards, it could potentially help with enforcing violations.

INTRODUCTION

Cyanide fishing is a destructive fishing technique widely used to capture live coral reef fishes, including species destined for the marine aquarium and live reef food fish trades. The use of cyanide on coral reefs was first documented in the early 1960s in the Philippines to capture aquarium fishes, principally for export to the United States, the United Kingdom, Germany, and France. Cyanide fisheries expanded to the live reef food fisheries in the 1970s, and over the next two decades it spread throughout Southeast Asia and into the Pacific islands. Cyanide fishing has been confirmed in at least 15 countries, including Indonesia, Malaysia, Maldives Islands, Papua New Guinea, the Philippines, Sri Lanka, Thailand, and Vietnam (Jones et al., 1998).

Most commonly, sodium cyanide is dissolved in seawater in plastic squirt bottles. Divers using hookah squirt the milky solution at the target fish, which then often retreat into crevices in the reef or within coral thickets. These corals may be subsequently broken apart by the diver to capture the fish. Cyanide tablets may also be secured to sticks and held close to a fish, or cyanide is mixed with bait and thrown overboard or placed into fish traps. There are also reports that fishermen occasionally pump the cyanide into the water from surface boats, mainly to target grouper spawning aggregations. The stunned fish are then captured with hand-nets or attached to lines and hauled to surface support boats, where they may directly enter the trade or be held in floating cages until export.

Cyanide is used in two very different live reef fisheries. The Live Reef Food Fish Trade (LRFF) regularly targets large groupers, coral trout, barramundi cod, and humphead wrasse. Although it focuses mainly on a small number of species, the actual trade is large in terms of the biomass of fish collected. At its peak in 1997, the volume of fish in trade was estimated at about 50,000 metric tons. More recently, trade is about 30,000 metric tons per year, with about 60% imported into Hong Kong and the remainder destined for mainland China, Taiwan, Japan, and other Asian markets. Until the 1970s, LRFF fisheries were mainly confined to areas in the South China Sea in close proximity to ports in Hong Kong and mainland China. The trade spread from the Philippines to Indonesia in the 1980s, and continued to expand to countries in the Pacific Ocean and Indian Ocean during the 1990s. Cyanide use has followed the expansion of the LRFF trade. One reason for the rapid expansion of this trade is that live fish can fetch substantially higher prices than dead fish of the same species. The total retail value of the LRFF was around \$350 million per year between 1997 and 2001. By 2002, it increased to about \$486 million for Hong Kong and \$810 million for the entire trade. Individual fish can sell for up to \$180 per kilogram, depending on species, taste, texture, availability, and time of year.

Unlike the LRFF, the Marine Aquarium Trade (MAT) consists of a high diversity of fishes, most of which are taken from the wild. Over 1,400 species of reef fish are traded worldwide for home aquaria at an annual volume of about 30 million fish, with approximately 16 million imported each year into the United States. Between 70 and 80% of these fish are from Indonesia and the Philippines, where cyanide use is most prevalent. In the MAT, species targeted by cyanide fishing include nearly all coral fish species, but its use may be most prevalent among

the high-value fish species such as emperor angel-fish (*Pomacanthus imperator*), blue surgeon-fish (*Paracanthurus sp.*), and blue ring angelfish (*Pomacanthus annularis*) (Fahrudin, 2003). The marine aquarium industry worldwide is worth an estimated \$200 to \$330 million annually (USCRIF, 2000. Wabnitz et al., 2003. FAO, 1996-2005). When examined by weight, aquarium fish are valued at \$500 per kilogram or more, which is considerably higher than a similar weight of food fish (Cato and Brown, 2003. Wabnitz et al., 2003).

Environmental concerns about cyanide fishing

Although illegal in most countries, the use of cyanide to capture live reef fish remains pervasive, propelled by the lucrative growing and largely unregulated international trade in live reef food fish and the marine aquarium industry. The United States is the number-one importer of coral reef fish for the aquarium trade, and the demand for these species may be one factor driving the continued use of cyanide. Cyanide is toxic to fish because it interferes with oxygen metabolism by blocking the key enzyme system, cytochrome oxidase (Metzler, 2001), and blocks enzymatic pathways in the liver (Solomonson, 1981). Once inside the fish tissue, cyanide reacts with thiosulfate to produce the comparatively nontoxic thiocyanate which is excreted in the urine. Rapid detoxification enables animals such as fish to ingest high, sub-lethal doses of cyanide (Eisler, 1991), although some of the effects are irreversible and may lead to the death of the fish (Way et al., 1988).

Several studies have also demonstrated negative impacts of cyanide on non-target coral reef species including corals (Cervino et al., 2003. Jones et al., 1998). Exposure of corals to cyanide causes rapid signs of stress and bleaching, and at high concentrations, progressive tissue sloughing that can lead to colony mortality. Fishermen spray cyanide into crevices and coral thickets where fish often hide, and then break apart the corals to access the stunned fishes, leading to substantial damage to the habitat. Large percentages of the target fish captured with cyanide die during collection or in transit due to their weakened state, which requires fishermen to capture significantly higher numbers of fishes than would otherwise be needed. In fact, some studies indicate that as many as 75% of fish collected with cyanide die within hours of collection, and another 30% die prior to export. In addition, more than half may die shortly after arrival in the United States from a combination of the poisons used in the capture and stress associated with handling and transport. Cyanide fishing is also risky for the divers, who often go to considerable depths for extended periods without following proper dive procedures.

Conservation approaches to address cyanide fishing

Although cyanide fishing is illegal in most countries, poor law enforcement capabilities and high levels of corruption have allowed the use of cyanide to continue. In 1989, the Haribon Foundation and Ocean Voice initiated a program to train fishermen in the use of nets as an alternative to cyanide. A second, more aggressive program was implemented in the Philippines in the early 1990s by the International Marinelife Alliance (IMA), in partnership with the Philippine government's Bureau of Fisheries and Aquatic Resources. Through a combination of the right policies and laws, improved enforcement, enhanced public awareness, training of cyanide fishers in cyanide-free fish capture techniques, development of livelihood alternatives, community-based resource management programs, and cyanide testing of live fish exports

through the implementation of a network of cyanide detection laboratories (CDT labs), this program successfully reduced cyanide fishing within the Philippines, at least temporarily. Over a period of roughly eight years, IMA tested 48,000 aquarium fish and food fish for the presence of cyanide. Cyanide was detected overall in about 25% of all aquarium fish and 44% of the food fish. The testing appeared to serve as a deterrent, at least in the initial years, as the proportion of aquarium fish testing positive declined from about 43% in 1996 to 8% in 1999. Unfortunately, the numbers of fish testing positive for cyanide has increased in recent years, and most CDT labs were closed in the mid 1990s.

In the United States, the issue of unsustainable trade in coral reef species was first highlighted in 1998 with Executive Order 13089 on Coral Reef Protection, which called for the creation of the U.S. Coral Reef Task Force (USCRTF). Over the next two years, USCRTF members developed a road map for coral reef conservation, the National Action Plan to Conserve Reefs, which outlines the key threats affecting reefs and identifies specific actions to mitigate those impacts. The Plan includes a section on international coral reefs and the role of the United States as a major consumer of coral reef species. This section identifies seven key strategies to advance a sustainable marine ornamental fishery, one of which specifically calls on the United States to improve enforcement capacity domestically and internationally. As one step to implement the recommendations in the Plan, the NOAA Coral Reef Conservation Program provided funding in 2003 and 2004 to reestablish a lab in the Philippines and expand testing into Vietnam, Indonesia, and Malaysia. These projects were halted in 2006, due to recent publications by researchers from the University of Hong Kong (Mak et al., 2006) and reports from the Marine Aquarium Council that suggested the Ion Selective Electrode (ISE) method used in the Philippines was not sensitive enough to determine cyanide traces in exposed fish.

The USCRTF has also adopted three resolutions on trade since 2004. The most recent, in May 2006, requested that the U.S. government identify existing or potential cyanide detection tests that could determine whether fish had been exposed to cyanide and develop a cyanide fishing mitigation strategy. In response to the USCRTF resolution, NOAA convened the International Cyanide Detection Testing Workshop (February 6-8, 2008, in Orlando, Florida) to advance cyanide detection methods for the live reef fish trade. The primary objective of this workshop was to review the state of testing methods and identify simple, cost-effective, rapid, and internationally accepted tests to detect cyanide or its metabolites at different points along the supply chain, from point of collection and export to ports of import in the United States. Also identified were key research needs and major steps that need to be implemented in importing countries and exporting countries to curtail the use of cyanide. The United States is committed to continuing its work with partners to address the recommendations identified in the workshop, including science questions pertaining to cyanide kinetics, additional testing and validation of cyanide detection methods, and, ultimately, implementation of cyanide detection laboratories.

TERMS OF REFERENCE FOR WORKING GROUPS

Working Group 1

The Field Forensics and Toxicology Working Group

Goal 1: Evaluate existing or potential analytical cyanide detection methods or tests and their applicability to marine fish.

- Develop a table of all cyanide detection methods. See draft format.
Include tests for:
 - cyanide by-products
 - biomarkers
 - adducts
 - morphological changes in tissues
- Provide assessment of existing knowledge of residence time of cyanide in marine fish
- Summarize methodology, equipment needs, costs, time required for testing, necessary scientific expertise, reliability and sensitivity. See draft spread format

Goal 2: Provide recommendations on the most practical, reliable, and user-friendly tests that could be used in marine fish.

- Provide recommendations for each test with respect to ability to be internationally accepted in a court of law
- Provide recommendations for each test with respect to ability to use test at collection sites and holding facilities (within hours of collection and with minimal technology)
- Provide recommendations for each test with respect to ability to use test at export sites (within days of collection and with minimal technology)
- Provide recommendations for each test with respect to ability to use test at collection sites and holding facilities (up to 1-2 weeks or more after initial exposure to cyanide)
- Provide recommendations for use of tests that determine cyanide presence versus a quantitative determination of cyanide concentration

Goal 3: Identify research needs to refine methodologies and/or develop other testing approaches.

- List of recommended research to conduct

Working Group 2

The Export Working Group

Infrastructure

Goal 1: Outline what to include in a robust scheme for monitoring exports to determine whether a fish was caught using cyanide.

- Provide an outline for ideal testing infrastructure including:
 - locations of offices and labs
 - staffing
 - budget
 - equipment
 - training
- List existing cyanide detection facilities
- List locations that do not, but should have cyanide detection facilities
- List names of government bodies involved in each countries' cyanide detection

Implementation

Goal 2: Identify steps, agreements, and/or partnerships that could be established between exporting governments, the U.S. government, NGOs, certification agencies, community groups, and academic scientists to implement cyanide testing.

- List existing partnerships related to cyanide detection
- List the five most important entities (NGOs, governments, scientists) in export countries that must be involved in establishment of revised cyanide detection system
- Briefly describe five most important areas to improve in existing detection system
- List alternate methods and programs that increase non-destructive catch practices

Enforcement

Goal 3: Provide recommendations with respect to the possibility and/or benefits of a mandatory, government-issued, export certification that states the fish were caught with legal practices.

- Provide a brief description of requirements to enforce and or prosecute the use of illegal fishing practices in export countries
- Provide recommendations regarding legal efficacy of a test that identifies the presence of cyanide versus a test that provides a quantitative estimate of cyanide concentration

Working Group 3

The Import Working Group

Infrastructure

Goal 1: Outline what to include in a robust scheme for monitoring imports to determine whether a fish was caught using cyanide.

- Provide an outline for ideal testing infrastructure including:
 - locations of offices and labs
 - staffing
 - budget
 - equipment
 - training
- List locations that do not, but should have cyanide detection facilities
- List names of government bodies involved in each countries' cyanide detection

Implementation

Goal 2: Identify steps, agreements, and/or partnerships that could be established between exporting governments, the U.S. government, NGOs, certification agencies, community groups, and academic scientists to implement cyanide testing.

- List existing partnerships related to cyanide detection
- List the five most important entities (NGOs, governments, scientists) in import countries that must be involved in establishment of revised cyanide detection system
- Briefly describe five most important areas to improve in existing detection system
- List of alternate methods and programs that increase non-destructive catch practices

Enforcement

Goal 3: Provide a recommendation with respect to the possibility and/or benefits of a mandatory, government-issued, export and/or import certification that states the fish were caught with legal practices.

- Provide a brief description of requirements to enforce and or prosecute the use of illegal fishing practices in import countries
- Provide recommendations regarding legal efficacy of a test that identifies the presence of cyanide versus a test that provides a quantitative estimate of cyanide concentration
- Provide recommendations regarding legal efficacy of a tiered approach to testing
- Discuss the current procedure for examination of imports of wildlife, and how this varies depending on international regulations (e.g., CITES)
- Respond to the following questions:
 - If an exporting country requires documentation verifying the absence of cyanide, can import countries such as the U.S. prohibit imports of shipments without appropriate certificates?

- Can the U.S. require verification that no illegal fishing practices were used to capture the fish included in a shipment under existing legislation? If not, what is needed to prevent imports of cyanide caught fish? Can the burden of proof be on the exporter/importer?
- What would be needed to prosecute violations under the Lacey Act?
- Are there provisions under Title IV section 403 of the Magnuson Act that can help eliminate imports of cyanide caught fish? What requirements do we have under Magnuson to address illegal, unreported, or unregulated fishing?
- What would we need to implement prohibitions on imports of aquarium fish for exporting countries that cannot verify that they are engaging in sustainable harvesting practices? How would we make these import restrictions consistent with both domestic legal requirements and WTO obligations?

Report from Working Group 1

Participants in the Field Forensics and Toxicology Working Group focused their discussions on five key topics:

- 1) Evaluating different cyanide detection methods that are currently available to test for cyanide and cyanide metabolites;
- 2) The feasibility of applying these methods to detect cyanide exposure in ornamental reef fish under different export and import scenarios;
- 3) Type of training and quality control that would be necessary to ensure consistent, reproducible results;
- 4) Modifications or additional research that would be necessary to apply existing tests to ornamental fish at points of collection, export and import; and
- 5) Research necessary to characterize natural levels of cyanide and patterns of uptake and metabolism of cyanide in marine fishes.

Goal 1: Evaluate existing or potential analytical cyanide detection methods or tests and their applicability to marine fish.

The Working Group identified and reviewed numerous methods that have been used to detect cyanide and cyanide metabolites. The most common of these include: electrochemical methods, ion-selective electrodes, spectrophotometric and fluorescence methods, chromatographic techniques and biomarkers of cyanide exposure (Table 1). While these are widely used for determining cyanide concentrations in water and in certain biological fluids such as blood, very few of these have been applied to marine fishes, and even fewer have been successfully used on fish tissue.

In order to effectively test for cyanide in marine fish, particular aspects of testing should be addressed. The appropriate method will depend on the point in the chain of custody (e.g., point of collection, export or import), as this will dictate the particular analyte being tested (cyanide vs. thiocyanate and ACTA) and the type of sample being analyzed (sample matrix - blood, tissue or organs). The sample matrix will dictate the amount of sample preparation and cleanup required, while the analyte concentration dictates the sensitivity requirement of the method. The sensitivity of a method may be further influenced by sample preparation and interfering substances and the instrumentation used.

Cyanide detection methods can range in procedure, extent, and methodology. Presented here is a brief summary of the approach, benefits, and limitations of known testing methods:

Electrochemical methods

Electrochemical analysis of cyanide and thiocyanate concentrations in biological samples, including plasma, tissue, and whole blood have been conducted using ion-selective electrodes (described below), potentiometry, amperometry, polarography and coulometry. Two clear benefits of these methods are high and quick analysis time. However, they can be subject to multiple interferences from many organic and inorganic ions, including sulfide, Fe^{3+} , ClO^+ , NO^2- , N^3- , and I^- .

Polymeric membrane based ion selective electrodes (ISE)

The American Society of Testing and Materials (ASTM) ISE method is the only approach that has been widely used to detect cyanide exposure in marine fishes. The method involves an acid digestion of the fish to liberate hydrogen cyanide gas, and capture of cyanide ions in sodium hydroxide solution after reflux distillation. Chemicals must be added to help remove interfering substances such as chlorine and hydrogen sulfide. An ISE meter manufactured by Thermo-Orion, using a membrane made of AgI or Ag₂S, is then used to analyze cyanide concentrations, based on its interaction with silver. ISE methods may also work for the detection of thiocyanate.

Spectrophotometric and fluorescence methods

Both spectrophotometric and fluorescence methods require extraction techniques to isolate cyanide and eliminate interferences from blood. Spectrophotometric methods have been used for detection of cyanide, thiocyanate and ACTA. One common approach involves the König dye synthesis to form a cyanide halide that is reacted with an aromatic amine to produce a glutamic aldehyde product that is measured in the visible region of the spectrum. Spectrophotometric methods have adequate sensitivity, but they may lack specificity due to interferences from other chemical species commonly present during the analysis of cyanide, especially thiocyanate and thiosulfate. They also require lengthy preparation times and the products may be unstable. A number of fluorometric assays are available to determine cyanide, which have several advantages over spectrophotometric methods, including a lack of interference from thiosulfate and greater sensitivity.

Flow injection analysis (FIA)

The FIA method is a simple one with high reproducibility. The approach involves injecting a sample solution containing the target molecule into a flow tube where it reacts with certain chemicals. When the products reach the detector, the target molecules in the sample are measured. The user is able to control the measuring conditions precisely and also has the capability to continuously measure cyanide.

Biosensors for detecting cyanide ion

These methods primarily evaluate chemical reaction products based on the enzyme inhibition of cyanide, cyanide degrading enzymes, and microbial sensors which measure oxygen uptake by bacteria, yeast or other microorganisms. Biosensors have a rapid response, high selectivity, and a pollution free procedure. Most biosensors have the advantages of being portable, low cost, easy to use, and high selectivity. These methods, however, rely on chemical and physical procedures that can be slow, complex, and require the use of expensive equipment and environmental loading reagents. Other limitations of biosensors include degradation of the biological components that make up these sensors, inconsistent electrochemical signals, and difficulty producing sufficient quantities and activities of enzymes or microbes on which these sensors depend.

Recently, one group applied a biosensor approach to marine fishes. Organs of the fish were homogenized with NaOH and a fungal enzyme extract was used to produce formate from metal-cyanide complexes; the formate was converted using an enzyme to NADH which was

measured spectrophotometrically. This approach has not yet been applied to thiocyanate or ACTA.

Chromatographic methods

Gas Chromatography (GC), high performance liquid chromatography (HPLC), and ion chromatography (IC) can all be used for determination of cyanide. These methods employ electron capture detectors, electrochemical detectors, UV/VIS detectors, fluorescence detectors, conductivity detectors, or amperometric detectors. Three types of liquid chromatography have been used to analyze cyanide: reverse-phase high-performance liquid chromatography (RP-HPLC), ion chromatography (IC), and capillary electrophoresis.

Liquid chromatographic techniques can determine trace amounts of an analyte and can efficiently separate analytes from interfering components in the matrix, offering advantages over spectrophotometric, luminescent, and electrochemical methods. Liquid and gas chromatographic techniques also have the ability to simultaneously analyze for cyanide and thiocyanate. IC methods can determine all species of cyanide by separation. This technique obviates the need for distillation to convert cyanide complexes from metal to HCN. A number of pretreatment steps have been developed to facilitate the analysis of cyanide, thiocyanate, and ATCA using GC. For example, the sampling of cyanide from the sample head space is the most common pre-analysis step when using the GC method.

Simple field test

A number of “dip stick” type tests have been developed, primarily for detecting cyanide in water (e.g., the Cyanide ReagentStrip™ Test Kit). These include colorimetric kits that are dependent on color changes or based on ion selective electrodes (ISE) linked to portable ISE meters. While some experimentation has been undertaken to modify these tests for the detection of cyanide in fish, nothing conclusive has been developed to date. For example, cyanide could be detected in fish tissue that had been digested in concentrated NaOH using the *Soudararajan Procedure*, but the test appears to give anomalously high cyanide readings possibly due to interference by sulfide. An alternate approach may include digestion of fish in sulfuric acid in a closed container, with measurement of cyanide liberated as gas using test strips.

Goal 2: Provide recommendations on the most practical, reliable, and user-friendly tests that could be used in marine fish.

The Working Group discussed the practicality of each test, including the cost and equipment needs, level of expertise necessary, time required to analyze samples, reliability and reproducibility of different tests, and feasibility of implementing testing in either an exporting or importing country. Other considerations included factors that may affect the results, such as cellular absorption and detoxification kinetics, sampling and analysis time, sample storage time and conditions, and the sample matrix.

Participants also discussed the need for the development of new methodologies or modifications that could be made to existing tests that would be necessary under different scenarios to address 1) testing of small fish (fish tissues) instead of blood; 2) testing of cyanide versus thiocyanate and ACTA; and 3) benefits and limitations of rapid tests that could be used to detect the presence of cyanide or cyanide metabolites instead of the actual concentration.

Timing of testing

The working group members could not come to consensus on the amount of time cyanide remains detectable in fishes. Several participants suggested that cyanide is quickly metabolized and excreted in a matter of hours, while others maintained that cyanide is retained for longer time periods.

At present, there is considerable information on metabolism of cyanide in blood. When analyzing cyanide from blood (the preferred method for determination of cyanide exposure in larger species), analysis must occur as soon as possible after exposure since the concentration of cyanide in the blood decays within minutes to hours. However, this may not be the case for fish tissue, as there is evidence that cyanide is concentrated in several organs and tissues, where it is slowly converted to SCN. There are also reports of rapid excretion of cyanide and SCN in urine. It is important to note that most work done with fish has been on freshwater species, and the rate of excretion in saltwater fish is likely to differ since, being hypo-osmotic in relation to seawater, they have a lower rate of urine production.

The concentration of cyanide present in fish over time is dependent on the concentration of the cyanide solution used during exposure, the length of time of the exposure, time for holding and duration of transport, post-collection treatment, as well as other factors. Considerable work has been done on cytochemistry of cyanide in the rainbow trout (a freshwater fish) and linkages between cyanide exposure and conversion to SCN, but most of this work involved chronic exposure to low levels of cyanide instead of a single or pulse-dose of a much higher concentration as would be the case with marine fishes.

In general, all researchers found a progressive increase in SCN⁻ in plasma over multiple days, followed by a decline until a period where it was no longer detectable (from 16 days to 16 weeks or more). In addition, at least one study reported detectable levels of HCN in plasma after 20 days. According to one participant (Rubec), cyanide remained detectable in marine fish tissue up to 5-14 days after exposure when using the ISE method.

Type of sample

Most cyanide detection tests have been developed for analysis of cyanide in biological fluids in vertebrates such as blood, urine, and saliva, or in other aqueous solutions including water, with limited sampling of mammal and fish tissues and organs. While it may be feasible to obtain sufficient quantities of blood from larger fish collected for the LRFF trade, most marine ornamental fish are too small to obtain the volume of blood needed for sampling. As a result, cyanide testing must be performed on ground fish tissue or organs. This, however, may present additional complications including 1) interference associated compounds used to digest tissue, such as sulfuric acid, which may trigger oxidation of thiocyanate to cyanide; and 2) variations in cyanide concentrations between organs/tissue due to differential rates of metabolism and variations in the concentration of the enzyme that catalyzes conversion of cyanide to thiocyanate.

Substance being tested

To date, the only large scale testing for cyanide exposure in marine ornamental fish has been done at points of export using the ISE method to detect cyanide concentrations. However, because of concerns of rapid metabolism of cyanide, testing at points of import (and possibly points of export) may also require methodologies that can detect thiocyanate, ACTA or other metabolites, which is likely to present added challenges. For example, thiocyanate levels are normally quite high and can be inconsistent. Large variation in background thiocyanate concentrations reported from mammals would present problems when trying to quantify low level cyanide exposure, and large and variable concentrations of thiocyanate may indicate that thiocyanate is involved in a number of biological processes in addition to cyanide metabolism. One advantage of using ATCA is that it is stable in biological samples for months at freezing and ambient temperatures, and therefore may be a lasting signature for cyanide exposure. Unfortunately, there are few existing techniques to analyze ATCA from biological matrices and not many studies have evaluated the relationship between ATCA concentrations and cyanide exposure.

Quality control and validation of results

All of the participants recognized the importance of adopting testing methods that generate accurate and reproducible results. To be accepted in a court of law the results should vary minimally between testing methods and the chemist performing the test. These methods must demonstrate adequate sensitivity, specificity, accuracy, and precision as determined by the “fit for purpose” concept. There was considerable discussion about the possibility of incorrect results, as the amount of cyanide detected in a sample may be altered due to interference from inorganic and organic ions as well as the possibility of interconversion between cyanide, thiocyanate and other metabolites resulting from sample preparation and storage.

Once a test is adopted, training, quality assurance, and proficiency programs need to be implemented at cyanide testing laboratories and for field testing. Many of the CDT procedures require extensive training and chemists conducting these tests need to be certified and undergo periodic spot checks to ensure performance consistency and accurate test results. The Chair of the working group drafted a white paper which outlines a possible procedure for QA/QC. He suggested that a training program must be developed employing both didactic and

hands-on training that develops a demonstrated acceptable level of performance, as defined by the performance characteristics of the specific test and the needs of the analysis. A quality assurance program should be based on ISO 17025 or similar structures and should at a minimum employ documented Standard Operating Procedures (SOPs), bench and blind QC samples and statistically determined performance limits. A proficiency testing program should be conducted by an independent body and employ the use standard samples, unknown to the analyst, analyzed across the network and evaluated statistically to identify laboratory results that do not meet network performance standards. While there is a recognized need for QA/QC, one participant was very concerned about the cost and time necessary for the detailed validation of the results of the particular test chosen, and how this is likely to delay implementation of a network of CDT laboratories.

The best test

The overall conclusion of the group was that there would be a need for, at minimum, three to four different types of tests, depending on the point in the chain of custody where the fish are tested. There currently only exists a single test that has been demonstrated to work in fish, shortly after collection and for detection of cyanide only, although there are many available methodologies that could be modified to test for presence of cyanide instead of quantity and to quantify other cyanide analytes.

It would be extremely valuable to implement a rapid field test used at collection points. This test would need to verify the presence of cyanide, but not necessarily the actual concentration. Not only could it be used by enforcement officers as a deterrent, but it could also help identify particular fish or particular collectors suspected of using cyanide, with more detailed quantitative monitoring done on samples of concern. The preferred methodology is a rapid “dip stick” type test; however, additional research is needed on methods to extract cyanide from fish tissue to allow use of these approaches. At this point, this type of approach has not been successfully applied to homogenized fish tissue.

The ASTM ISE method was identified by all participants as the optimal choice for a test at points of export, mainly because it has been widely applied to marine fishes and it appeared to be effective during early phases of cyanide testing by IMA. However, several concerns identified in the peer-reviewed literature must be addressed before it (and subsequent certificates verifying fish are cyanide free) would be accepted by the United States. One participant stated that the method has undergone extensive round robin testing which addressed QA/QC, precision, and bias. He also pointed out why other researchers obtained conflicting results (e.g., they did not follow the methodology or they used a different electrode). Several other questions still remain such as 1) the possibility that fish exhibit background levels of cyanide but these could not be measured using the ISE method; 2) incomplete knowledge on how long cyanide is in fact detectable in fish; 3) lack of validation outside of the IMA/BIFAR network of the methodology used to digest fish and whether this results in incorrect readings due to interference of other substances.

A rapid “field” test would be needed for USFWS inspectors at points of import to verify the presence of cyanide and identify possible fish for more detailed laboratory analysis. This is not

currently available, and cannot realistically be developed until we address questions regarding the time after exposure cyanide and cyanide metabolites are detectable.

A quantitative method for laboratory analysis at points of import is needed for forensics laboratories. It is likely that the method would need to detect thiocyanate and not cyanide (or possibly ACTA or other analytes). Until more research is done, it is unlikely that an appropriate test for points of import can be identified.

Table 1: Summary of cyanide detection testing methods.

Detection Method	Testing For	Approximate Cost Low <\$5K Mod \$5-25K High >\$25K	Technical Training Required	Equipment Required	Sensitivity	Reliability	Samples/Day
Half-life	1 hour						
Location	Collection						
Headspace Test Strip	CN ⁻	Low	Low	Low	Poor	Unknown	Many
Location	Export						
Ion Chromatography	SCN ⁻	High	Mod-High	High	Good	Unknown	25+
Gas Chromatography	ATCA	Mod-High	Mod-High	High	Excellent	Good	25+
Colorimetric	SCN ⁻	Low	Low	Low	Good	Good	25+
Location	Import						
Ion Chromatography	SCN ⁻	High	Mod-High	High	Good	Unknown	25+
Gas Chromatography	ATCA	Mod-High	Mod-High	High	Excellent	Good	25+
Colorimetric	SCN ⁻	Low	Low	Low	Good	Good	25+
Half-life	10 hour						
Location	Collection						
Headspace Test Strip	CN ⁻	Low	Low	Low	Poor	Unknown	Many
Location	Export						
Distillation/ISE	CN ⁻	Low	Moderate	Moderate	Good	Good	<5
Ion Chromatography	CN ⁻ SCN ⁻	High	Mod-High	High	Good	Unknown	25+
Gas Chromatography	CN ⁻ ATCA	Mod-High	Mod-High	High	Excellent	Good	25+

Goal 3: Identify research needs to refine methodologies and/or develop other testing approaches.

The group summarized some of the pressing biological questions and information gaps needed before the most appropriate testing methods could be adopted. The consensus of the Working Group was that serious gaps exist in the understanding of the metabolism and elimination of the cyanide anion and its major metabolites in fish. Without a better understanding of these processes, the implementation of an exposure assessment method that would meet legal requirements would be difficult. Therefore, the highest priority for research involves support for a program that will generate the data necessary to determine the availability of cyanide and its metabolites for exposure assessment versus the time involved with the local collection of fish, their transport to a fixed site laboratory prior to export, and their arrival at the import destination prior to the implementation of an analysis method at any point in the delivery.

The concentration of cyanide present in the living fish will vary depending on the concentration of the cyanide solution used during exposure, the length of time of the exposure, time for holding and duration of transport, and post-collection treatment. Rates of metabolism may also vary depending on species or size. Research should encompass these possible variations, such that it is possible to quantify measurable levels of cyanide in representative species and life history stages of marine fishes, and how this changes over time, using at least two methods and multiple known concentrations. Participants also identified a list of additional research needs for developing and refining cyanide detection testing methods:

- Determine rates of metabolism of cyanide. A detailed analysis requires that the analyte is stable and at elevated levels in the fish long enough for it to be delivered to the laboratory and an examination conducted. Without a better understanding of these processes in representative marine fish species, the implementation of an exposure assessment method that would meet legal requirements would be difficult. The Working Group identified the highest immediate priority is research to determine the half-life of cyanide and major metabolites (especially thiocyanate) in marine fish, and variations between species, life history stages, and the biological matrix examined. To represent the time from initial collection and transport from the reef to holding facilities in exporting countries and then on to import destinations, fish samples should be exposed to varying concentrations of cyanide, with representative individuals sampled immediately after exposure and subsequent defined intervals.
- Identification of the presence of background levels of cyanide (and major metabolites) in marine fish. This would include an evaluation of different species collected from a variety of locations and whether cyanide levels are above the limit of quantification. One aspect should also include a determination of detectable background levels, using fish from the wild caught with nets as well as captive bred species. In addition, the effect of freezing samples must be determined for both field collection and delayed analysis samples to prevent misinterpretation of data obtained from frozen samples. These steps are critical to ensure that the applied test can detect elevated levels of cyanide which would be indicative of exposure by

fish collectors. To perform this research, validated methods for the determination of cyanide, thiocyanate and other metabolites (e.g., ATCA) in homogenized fish tissue must be used. These methods must demonstrate adequate sensitivity, specificity, accuracy and precision.

- Demonstration of adequate recovery of cyanide in fish samples that are digested and verification that sulfuric acid or other compounds used to digest the fish do not interfere with the readings.

Working Group 1 Participants

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Report from Working Group 2

Participants in the Export Working Group discussed current actions underway to address cyanide fishing, the effectiveness of these activities, and additional efforts that would be needed to successfully eliminate cyanide use for the capture of marine fishes. A comprehensive and robust scheme would require six strategic actions:

- 1) Education for fishermen and exporters in problems associated with cyanide use and a training program in the use of nets;
- 2) Improved partnerships between all sectors of the ornamental and live food fish trade, including industry, government agencies, and conservation groups;
- 3) Targeted cyanide testing in the field and at points of export;
- 4) Improved monitoring and validation of exports as cyanide-free;
- 5) Licensing schemes and adequate enforcement measures for offenders; and
- 6) Sustainable funding mechanisms to support cyanide detection labs.

The group also discussed some of the difficulties in implementing a cyanide testing network, pinpointing the largest gaps as insufficient funding, lack of trained personnel, and limited laboratory capacity. Funding is particularly challenging, as there are discrepancies in monitoring, testing, and enforcement under any cyanide use prevention scheme. They also identified difficulties associated with testing due to the large spatial and temporal scale over which the trade operates, and limitations of available testing methods.

The Working Group developed a short list of priorities for cyanide testing in export countries. The first priority is to validate the leading cyanide testing methods for accuracy. The chosen method must be a credible, proven test that is effective in prosecution. Once there is consensus on a valid, defensible test, training in testing procedures and cyanide-free catch techniques must take place. In conjunction, a database should be developed to track testing results and violations. This database should be linked to the accreditation and licensing scheme, with repeat offenders losing their license for collection or export, along with other penalties.

Goal 1: Outline what to include in a robust scheme for monitoring exports to determine whether a fish was caught using cyanide.

Considerations for testing

A successful detection test must be sensitive enough to detect cyanide or cyanide metabolites for a minimum of 5 to 10 days after collection to accommodate the time period between collection and export.

As the demand for live reef fish has increased and local fish populations have declined, areas of collection have expanded. Whereas most collection once occurred on local reefs near villages in close proximity to major international airports, collection areas now include remote offshore reefs. The average time between collection and transport to a village landing area is

estimated at roughly 3 to 5 days. However, in some locations roving fishermen may embark on collecting trips that last for up to 2 weeks before fish are finally landed in a local village. At holding facilities, aquarium fish are typically quarantined for 1 to 4 days before they are sold to a middleman or exporter. For live reef food fish, the time between collection and export can be even longer: collectors may hold fish in floating cages for several weeks before they are purchased by a middleman or exporter. Trade routes can also be quite complex, and may include transport via boat, road, local flights and international flights. Marine aquarium fish are also typically held by the exporter for an additional period of 2 to 5 days before shipping.

Optimal targets for cyanide testing

Identifying the most effective target points for cyanide testing is a priority. Participants thought the easiest location to test for the presence of cyanide was points of export at international airports, since the majority of fish are sent abroad by air. Cyanide detection testing (CDT) laboratories should be established close to major regional airports, including:

- Indonesia — Bali, Jakarta, Sulawesi, Ujung Pandang, Manado, Surabaya
- Vietnam — Saigon, Hanoi, Da Nang
- Philippines — Manila, Cebu

Also needed is development of a field test that could be implemented by enforcement officers at landing areas. As reef fish may be transported by land and boat (e.g., live reef food fish), random testing would ideally be done in every village with a landing area. This raises the issue of choosing the most appropriate methods for sampling. An effective cyanide testing regime must address who would be impartial samplers, how often to sample, what size sample to take, and whether to include targeted samples as well as random ones.

Obstacles limiting testing

Assuming the Ion Selective Electrode (ISE) cyanide test proves valid, additional obstacles to using the test centered on funding, geographic barriers and distance, and potential social backlash. Testing at field sites and airport points cover both ends of the export chain, and is therefore ideal. However, lack of funding might constrain this possibility. Funding for testing would initially rely largely on government support, possibly supplemented by international donors and importing countries. Longer-term financing may require inspection fees that are covered by the exporter as well as taxing particular industry components; this may subsequently be passed on to the importer and ultimately the consumer. Funding for testing schemes should be self-sustaining, and needs further consideration on how to structure and implement such a system on the export side.

Enforcement considerations

The process of accountability is a challenge. On one side is the issue of test verification, and on the other is the issue of prosecution. Creating a system for trustworthy, reliable testing must be airtight in order to stand up to exporters who challenge it, especially if it becomes a requirement. There must also be incontestable consistency, so that testing at two separate sites does not return differing results. Holding someone accountable would have to lie with the government, and depends heavily on where along the export chain you wish to target. There

must also be some degree of coordination between export and import nations for a truly successful testing system.

Monitoring fish at landing areas can also employ indirect evidence of cyanide use. For example, if fishers are catching more fish than would be expected under normal circumstances, they might be using cyanide. Further, certain species are more often caught using cyanide than others; therefore, monitors should be trained to know which species to look for.

Perhaps the most logical and proven method calls for placing the burden on exporters to clean up the supply chain, but it is important to note the inherent challenges in this type of approach. Testing only at points of export may be complicated, as the exporter may request cyanide-free fish but be unable to verify this. Because exporters may purchase fish from middlemen, and fish from multiple sources may be mixed within the export facility, it may be challenging to demonstrate the connection between the exporter and the collector. Despite these concerns, the participants agreed that initiating a cyanide detection testing scheme at export points remains the most practical and manageable starting point.

Target points

In addition to some of the limitations described above, the group felt additional measures are needed to tackle the actual root causes of the problem. Several participants felt that exporters were the cause of the problem, as opposed to the actual fishers. The exporters may supply the cyanide in some cases, and they also provide few incentives to collectors who use nets instead of cyanide. However, testing only at airports may be inadequate, as the volume of fish exported on a daily basis may exceed the limits of testing, thereby allowing many cyanide-caught fish to be exported. It is important to recognize that this oversight may occur even in the best of schemes. However, if sampling is frequent enough and penalties high enough, it should become more economical for exporters to invest in cleaning up the supply chain.

One other alternative could include spot checks at points of landing, solely to verify exposure to cyanide, followed by more quantitative testing of fish at airports, with repeat offenders losing their license (fish collectors) or being prosecuted (exporters). This would shift the burden to the exporters, who ultimately make most of the profit and has direct control over what they purchase from fishermen. In the past, the Philippines conducted monitoring at the landing areas, and samples were then sent on for further testing at the airport lab.

In conjunction with testing, educating collectors has been pushed in the Philippines, while Vietnam's approach to the similar issue of dynamite fishing has been to deputize villagers who curtail nighttime use and enforce local fisheries management regulations.

Goal 2 : Identify steps, agreements, and/or partnerships that could be established between exporting governments, the U.S. government, NGOs, certification agencies, community groups, and academic scientists to implement cyanide testing.

Harmonization

Export countries emphasized the need for harmonization of standards between importers and exporters to eliminate policy gaps and potentially tighten enforcement. Import countries should create clear requirements for shipments, make the policies available to exporting countries so they can comply, and make them understandable. Information on quarantine requirements, clear documentation requirements, and the creation of a unified labeling and packaging system are all ways to reduce discrepancies.

Training and education

Parties emphasized that tying education and enforcement together is critical to a successful implementation strategy. This includes educating government officials as well. Public awareness campaigns featuring celebrities or other popular personalities might help get the general public interested and galvanized on these issues. Education programs must be designed carefully and with appropriate follow-up, as past education efforts have also made users of cyanide more clever about covering their tracks. Re-education and training for collectors will be necessary if some are forced out of the trade by new enforcement and regulations. Parties should work further on eco-tourism and supplementary livelihood options.

Partnerships

Partnering with multiple stakeholders and interested parties will increase the likelihood of a successful cyanide testing regime. Importing parties should improve coordination and communication with importing countries. Data on exporters could be shared with government agencies in importing countries, including the numbers of legitimate exporters, which ones still use cyanide, where the fish are caught, and so forth. Sharing such information could help identify legal and illegal shipments for importing countries.

Because the marine aquarium fish trade is lucrative for exporting countries, parties should not overlook the role of business entities. Garnering support from the business sector could push government in the right direction, or achieve faster results than diplomatic avenues.

Partnering with local communities is extremely important. It is vital that community-based initiatives be carried out correctly. Improper training can result in the community becoming even more committed to hiding their cyanide use. Licensing is a potential avenue for community-based cyanide-free fishing efforts. In the Philippines, parties are re-defining things by province and talking to community leaders (mayors) for input on how to regulate live fish trade in terms of licensing.

Partnering with both local and international NGOs can provide transparency and aid in enforcement, education, and communication from the provincial level up through the national and international levels.

Goal 3: Provide a recommendation with respect to the possibility and/or benefits of a mandatory, government-issued, export certification that states the fish were caught with legal practices.

Cyanide detection: current procedures

Efforts to find cyanide in exporter countries are already taking place through boat searches using local enforcement or the Navy, and training on where to look for cyanide. However, uncovering cyanide use is harder now due to several key behaviors, including users traveling at night, cyanide stashes hidden underwater, and a switch from fishing out an area to collecting for two days and then moving on. Inspections already occur in Indonesia and the Philippines, but they are insufficient.

Limited cyanide testing is reportedly occurring at airports in the Philippines, but this testing may occur several days to two weeks after initial cyanide exposure. Due to the rapid metabolization of cyanide, this time frame may be too long for accurate testing. Sampling is either voluntary or provided by the exporter; there is no random or obligatory sampling for cyanide testing in Indonesia and the Philippines. Even with a sample provided, boxes may not get inspected before shipment. Also, if a shipment makes several stops before its final destination, the exporter has no control over the shipment at those stops, where shipments might be tampered with or altered.

Next steps

The Working Group felt the only viable strategy to accrue evidence of cyanide use hinges on a testing scheme; any other strategy would be difficult and, while it could be a supporting scheme, it could not be the main strategy. An effective test would need to detect prior exposure to cyanide at the landing area within a 5- to 7-day frame, and a 7- to 14-day frame at international airports. If the ISE test is examined and proven reliable, funding for reinstating and significantly enhancing the accompanying infrastructure will then need to be addressed.

Cyanide-free verification: testing outputs

The Working Group examined numerous options for verifying cyanide-free marine ornamental fish. Three main approaches were discussed.

- 1) Accreditation: The possibility of using a cyanide test as an accreditation tool may improve industry support as an alternative to a cyanide-free certification scenario. For example, if after 10 cyanide-free shipments occur, the company would be accredited for a year's time. Testing would continue even after accreditation, in order to maintain enhanced standards and monitoring.
- 2) Certification: The viability of independent third-party certification schemes is unclear at this point. Previously, shipments verified as cyanide-free based on the ISE testing method received cyanide-free certificates. While this could be a requirement for export of fish, it is not apparent whether this could be used as a legal toe-hold for prosecution between import and export countries. For example, if a shipment comes with a

certificate, but tests positive upon arrival in import countries, import enforcement officers can then testify in export countries.

- 3) Licensing: Under a licensing scheme, the marine resource oversight organization within each export country should determine loss of license for those who fail cyanide testing. Due to the contentious nature of prosecution, the scheme would need an independent group to do the sampling. However, this puts the pressure again onto the quality of the test (given that the law is clear). One suggestion was that the municipal government revoke the fishing license if authorities choose to trace it back to the point of collection. It was also suggested that, similar to anti-fencing laws, the government should see it as “anyone with stolen goods is guilty.” This would hold the exporter accountable if in possession of fish testing positive for cyanide.

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Report from Working Group 3

The Participants in the Import Working Group discussed the possibility of implementing a cyanide testing scheme at points of import, with an emphasis on the United States. Cyanide testing for this sector of the live reef trade would primarily address imports of marine aquarium fish (MAF), because the United States currently imports 50 to 60% of all MAF, whereas live reef food fish (LRFF) represent a small component of U.S. imports. While the trade in LRFF is substantial (up to 30,000 metric tons per year), most imports are destined for Hong Kong and other Asian markets. Testing options could differ between these two types of trade as well, as LRFF consist of much larger and differing, selective species than MAF. Hence, testing of imports of LRFF could focus on detection of cyanide in blood, while MAF testing relies on whole digested fish. The cytokinetics of cyanide may also differ among LRFF and MAF, in terms of rates of breakdown and removal, as cyanide can be removed from the blood much faster than from tissues. Larger, more active mobile predatory fish may have different rates of metabolism. Most MAF are small, site-attached species, whereas larger groupers and other species are likely to have a higher metabolism because they are faster swimmers. In addition, the background levels may vary considerably among taxa, and could be higher in larger LRFF as these are top predators that may be consuming smaller fishes containing cyanide.

Goal 1: Outline what to include in a robust scheme for monitoring imports to determine whether a fish was caught using cyanide.

The group suggested that implementation of cyanide testing at points of import would be much more difficult, costly, and time-consuming than at points of export. They identified numerous challenges that need to be addressed before an effective import cyanide testing scheme is possible. These include, but are not limited to, the following central issues:

Infrastructure and facilities

Deficiencies in staffing, funding, equipment, and facility resources remain key issues in developing a U.S. import cyanide monitoring system. At present, lead ports for fish shipments include Los Angeles and Miami, with some support from non-designated ports such as Charleston and Tampa. Hundreds of boxes of invertebrates, fish, and corals pass through U.S. ports daily (Table 1, and Figures 1-2). The U.S. Fish and Wildlife Service (USFWS) Law Enforcement staff largely focus on species listed under the Endangered Species Act (ESA) and the Convention on International Trade in Endangered Species (CITES), due to specific legislative mandates for these taxa. In addition, there are no adequate facilities to maintain large shipments of fish while testing is undertaken, and seizures of live fish imports would be impractical and likely to result in high levels of mortality.

Identification and testing

The United States currently imports about 16 million MAF per year. Shipments can range from 20 to 25 or more boxes for small tropical fish, while larger shipments can include 50 or more boxes. Each box may contain several hundred fish, with each fish packaged in an individual bag or with mixed species in larger bags. Opening the bags to examine a fish introduces oxygen out of the bag and further threatens survival. This poses a huge challenge for identifying all species and possibilities for cyanide testing. The optimal strategy for testing would include a rapid, simple diagnostic test for use at the port, such as a “dipstick” method, that would simply identify the presence of cyanide (“yes or no” system). The test cannot take more than a few minutes, based on sheer size of import shipments, lack of manpower, and split attention for ESA and CITES identified priority species. If the field test confirmed the presence of cyanide, a shipment could be seized and sent on to a diagnostic laboratory where a more detailed test could be performed to quantify levels of exposure.

Table 1: Shipments of marine ornamental fish in each United States port city between July 2004 and June 2005.

	No. of Shipments	% of Total Shipments	Quantity	% of Total Quantity
Agana, GU	7	0	1,865	0
Atlanta, GA	230	2	435,498	3
Baltimore, MD	1	0	45	0
Blaine, WA	2	0	16	0
Buffalo-Niagara Falls, NY	51	0	293	0
Chicago, IL	257	2	519,547	3
Detroit, MI	74	1	90,430	1
Dallas-Ft.Worth, TX	272	2	295,832	2
Honolulu, HI	1,492	13	584,755	4
Los Angeles, CA	5,548	47	8,994,755	56
Miami, FL	1,898	16	2,442,173	15
Newark, NJ	54	0	70,730	0
New York, NY	946	8	1,570,988	10
Portland, OR	78	1	51,950	0
Seattle, WA	73	1	54,689	0
San Francisco, CA	662	6	954,049	6
Sumas, WA	1	0	2	0
San Diego-San Ysidro	10	0	3,545	0
Tampa, FL	75	1	7,925	0
unknown	20	0	6,896	0
Totals	11,751	100	16,085,983	100

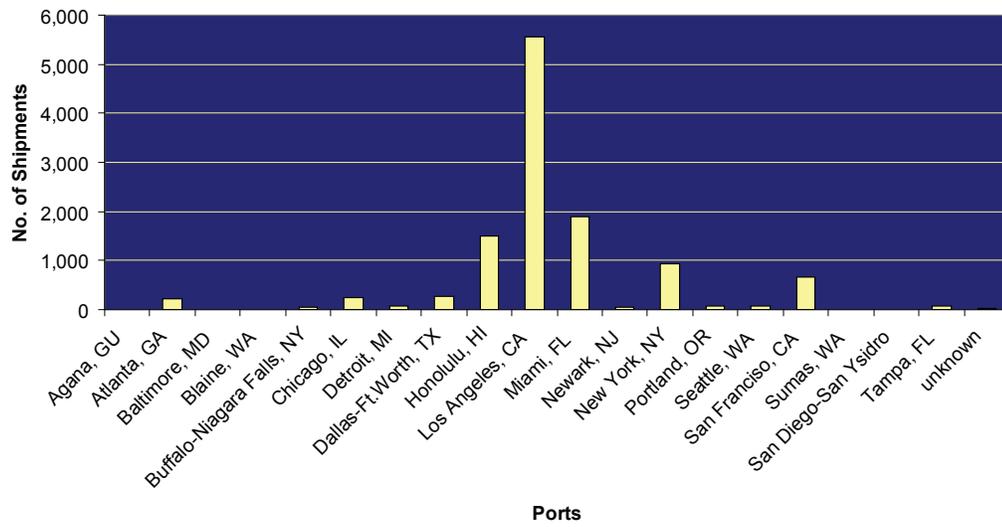


Figure 1: Number of Shipments by Port from July 2004 to June 2005.

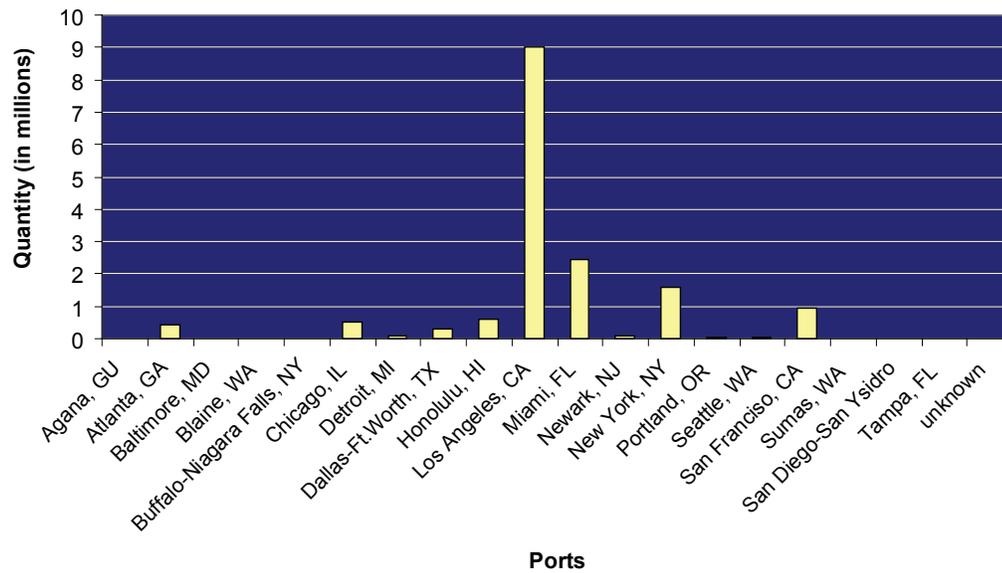


Figure 2: Quantity of Marine Ornamental Fish by Port from July 2004 to June 2005.

Goal 2: Identify steps, agreements, and/or partnerships that could be established between exporting governments, the U.S. government, NGOs, certification agencies, community groups, and academic scientists to implement cyanide testing.

Funding

Sufficient funding continues to be a serious concern across the board. Addressing existing obstacles and implementing new plans must have financial backing to move forward. One approach to enhance funding focuses on improved education and awareness about the issue and the factors limiting the ability of USFWS to effectively prevent imports of cyanide-caught fish. One recommended method involves the use of high-profile celebrities as spokespeople, which is likely to raise public concern and ultimately push politicians to alter and enforce policies on the aquarium trade. With political power behind them, trade programs could receive targeted funding through congressional earmarks and increased support from large donors and NGOs.

Harmonization

The participants emphasized the need for harmonization of standards between importers and exporters to eliminate policy gaps and potentially tighten enforcement. Import countries should create clear requirements for shipments, make the policies available to exporting countries so they can comply, and make them understandable. Information on quarantine requirements, clear documentation requirements, and the creation of a unified labeling and packaging system are all ways to reduce discrepancies.

Government linkages: exporting countries and importing countries

Exporting parties should improve coordination and communication with importing countries. Representatives from Vietnam, Indonesia, and the Philippines indicated that data on exporters could be provided (including the numbers of legitimate exporters), which ones still use cyanide, where fish are caught, and so forth. Sharing such information could help identify legal and illegal shipments for importing countries. United States enforcement staff would benefit from knowing which companies repeatedly test cyanide-free, as well as those companies that tend to use cyanide. Some degree of coordination must occur between export and import nations for a truly successful testing system.

Goal 3: Provide a recommendation with respect to the possibility and/or benefits of a mandatory, government-issued export and/or import certification that states the fish were caught with legal practices.

Export certification

Although participants from exporting countries suggested the possibility of either government-issued or third-party certificates verifying fish as cyanide-free, the United States is unlikely to use these for enforcement or prosecution. A certification document would not resolve or relieve any of the enforcement issues discussed, and did not seem reliable or establish any measure of relief that certified fish would be cyanide-free. Third-party certification also seemed improbable, as this process is most often used on the consumer side rather than the government side.

Current U.S. procedure for import inspections examination

All wildlife imported into the United States, with a few exceptions, must be declared to the U.S. Fish and Wildlife Service (USFWS) and cleared prior to release by U.S. Customs and Border Protection. Each shipment imported into the United States requires the importer and/or broker to file a U.S. Fish and Wildlife Declaration (Form 3-177) to the USFWS at the time of import. Each shipment includes the filing of the USFWS declaration form, which provides all relevant information pertaining to the shipment, such as date of import, U.S. importer, foreign exporter, the common and scientific name for each species of wildlife, quantity and origin of species, carrier, number of cartons containing wildlife, etc., and other accompanying documentation.

All shipments containing species listed under the Endangered Species Act (ESA), the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), or other federal wildlife laws must include the appropriate permits. As such, these types of shipments usually have a higher priority for physical inspection as opposed to shipments containing non-protected wildlife. The physical inspection entails verifying the shipment's contents with the information listed on the permits, invoices, and other documentation. Shipments containing non-protected species of wildlife are randomly inspected.

Due to the high volume of wildlife shipments imported into the United States, the mission and priorities of the Office of Law Enforcement, and limited staffing and resources at the present time, it is unlikely that all shipments containing live marine ornamental fish can be inspected and tested for the presence of cyanide unless new legislation is adopted and staffing is increased.

Policy changes and trade restrictions

The United States currently has no legislation banning the import of unsustainably caught aquarium fishes. It was generally agreed during the working group session that existing U.S. laws under the Lacey Act could be sufficient for prosecution, but the execution, funding, and support for those regulations needs consideration. Within the United States, the Lacey Act drives import inspection and enforcement. If exporting countries impose clear laws against exporting cyanide-caught fish, the United States could potentially prosecute violations under

the Lacey Act. Such legislation would allow the United States to demand more culpability on the importer and, in effect, make the trade of cyanide-caught fish an international violation.

Unfortunately, U.S. courts are unlikely to accept any analytical procedure that does not conclusively determine both the presence of cyanide and the amount of cyanide in the specimen. For example, if only trace amounts of cyanide are detected, the defense could offer expert witness testimony that cyanide can be found as a “background” contaminant in many ocean waters of the world.

Working Group 3 Participants

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WHITE PAPERS

Methods for Detection of Cyanide and its Metabolites in Marine Fish

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Introduction

Since the early 1960's, coral reefs have been increasingly exploited by fishermen-who use cyanide to capture reef fishes alive, in order to sell them to the live food fish trade (sold to restaurants in Hong Kong and mainland China) and marine aquarium fish trade (Rubec 1986, Rubec 1987). Fishermen stun the fish by squirting hydrogen cyanide (HCN) solution onto coral heads and into crevices in coral reefs. The sodium cyanide (NaCN) tablets used by collectors in the Philippines and Indonesia weigh about 20 g each (Johannes and Riepen 1995). Fishermen who collect ornamental fish for aquarium tanks generally place one or two 20 gram tablets of sodium cyanide (or potassium cyanide) into a one-liter plastic squirt bottle filled with seawater, while food-fish collectors use three to five tablets (Rubec et al. 2001). The tablets sequentially dissolve in the squirt bottle as collectors proceed to spray the reefs, making it difficult to determine the cyanide ion (CN⁻) concentrations being applied. It has been suggested that fish collectors use concentrations ranging from 1,500 to 120,000 mg/L (Johannes and Riepen 1995; Barber and Pratt 1997, 1998; Pet and Djohani 1998; Jones et al. 1999). Since not all of the cyanide applied from squirt bottles dissolves, it is sometimes visible underwater as a whitish plume (Rubec et al. 2001). Not all fishermen limit themselves to squirt bottles. Reports from the Philippines assert that 55-gallon

drums of cyanide have been dumped onto reefs to kill and capture food fish (del Norte et al. 1989, Johannes and Riepen 1995).

As far as cyanide testing is concerned, the amount of cyanide that might be detected is partly a function of the exposure concentration of cyanide. If higher doses are used in collecting, then higher initial concentrations in the fish are possible. The review conducted by SeaWeb at the CDT Workshop quoted a paper by Fahrudin (2003) that stated: "One cyanide tablet (2 g) is mixed with approximately 3 liters of water in a plastic bag or bottle" (SeaWeb 2008). Based on this information, Fahrudin (2003) calculated the resulting cyanide concentration as approximately 6.67 mg/L (ppm). It should be noted that the tablets weigh approximately 20 grams (not 2 grams) and that the tablets are usually dissolved in a squirt bottle of about one liter volume (Johannes and Riepen 1995). The minimum exposure concentration squirted on a fish on the reef exceeds 1500 mg/L (Cervino et al. 2003).

At the Cyanide Detection Workshop (held February 6-8, 2008 in Orlando, Florida) a subgroup of experts (Dr. Brian Logue, Dr. Robert Kobelski, Dr. Peter Rubec, Dr. Martin Frant, Ms. Benita Manipula) met to discuss the various methods that have potential for measuring cyanide or other metabolic byproducts in marine fish.

Pharmaco-kinetics of Cyanide In Fish

Dr. Kobelski of the Centers for Disease Control and Prevention (CDC) led the discussion. He directed the discussion toward the need to determine uptake and release rates for cyanide ion (CN^-) and/or the thiocyanate anion (SCN^-) in marine fish. The committee was divided between those that believe that cyanide is quickly metabolized and excreted in a matter of hours (Kobelski and Logue) and those that believe that cyanide is retained for longer time periods (Rubec, Manipula, and Frant). Dr. Logue of South Dakota State University has summarized information from mammals that indicates that cyanide in humans is quickly metabolized (Logue and Hinkens 2008). Dr. Kobelski noted that he had contacted a marine toxicologist at Cornell University that told him there was no reason to believe that fish would be different from mammals, since the pharmaco-kinetic processes are similar. The committee agreed that CN^- is concentrated in certain organs where it is converted to SCN^- by the enzyme rhodanese. While there are several pathways for cyanide metabolism, most of the SCN^- is excreted in the urine. There was disagreement concerning how quickly these processes occur in fish.

Dr. Rubec discussed the available research pertaining to fish. He also noted that the IMA was able to detect cyanide in marine aquarium fish (MAF) 5-14 days after they were captured using cyanide. This suggested that cyanide is retained in MAF for longer time periods than what has been documented with mammalian research (mostly humans).

There has been research conducted concerning cytokinetics of cyanide in freshwater fish (rainbow trout) but almost nothing published for marine fish. Dr. Rubec mentioned the research on rainbow trout by Raymond et al. (1986) and Brown et al. (1995). This research

has been reviewed in the paper concerning the cyanide detection testing (CDT) conducted by the International Marinelife Alliance (IMA) under contract with the Philippines Bureau of Fisheries and Aquatic Resources (BFAR) from 1993 to 2001 (Rubec et al. 2003).

The concentration of cyanide present in the living fish over time is dependent on several factors e.g., concentration of the cyanide solution used during exposure (squirting), the length of time of the exposure, time for holding and duration of transport, post-collection treatment (changes of water etc.). The conversion rate of hydrogen cyanide (HCN) to SCN^- appears to be limited by the availability of sulfur (Leduc 1984). Hence, both HCN and SCN^- occur in the blood.

The link between waterborne CN^- exposure and plasma SCN^- has been established in the rainbow trout (*Oncorhynchus mykiss*) (Raymond et al. 1986; Speyer and Raymond 1988; Heming et al. 1985; Heming and Blumhagen 1989; Lanno and Dixon 1993, 1996a, 1996b). In situations of chronic cyanide exposure, detectable levels of SCN^- were present in the plasma of trout after one day of exposure to 0.01, 0.02, or 0.03 mg HCN/L and increased over the 20-day exposure period (Raymond et al. 1986). Raymond et al. (1986) found that there was still enough free HCN present in the blood to inhibit the action of cytochrome oxidase in the liver of rainbow trout during 20 days of tests. Lanno and Dixon (1996a) also observed the accumulation of SCN^- in the plasma of trout exposed to 0.006 or 0.03 mg CN^- /L over a 16-week period. Bioconcentration factors (SCN^-/CN^-) were 170 and 88, respectively, for the two exposure concentrations.

Brown et al. (1995) used high-pressure liquid chromatography (HPLC) to examine the pharmaco-kinetics of plasma SCN^- in

rainbow trout exposed to 40 mg SCN⁻/L. Using depuration rate constants (k_2) ranging from 0.29-0.34 day⁻¹, a depuration half-life of about 4 days was estimated. Significant levels of SCN⁻ were detectable at 8 days and declined to below detection limits by 16 days. The determination of plasma SCN⁻ levels may be a useful biomarker of cyanide exposure.

The studies by Brown, Lanno, and Dixon cited in the previous paragraphs generally involved exposing rainbow trout to low levels of either CN⁻ or SCN⁻ for long time periods (chronic exposures). Research is needed in which fishes are pulse-dosed with higher concentrations of cyanide over short (acute) time periods (less than 90 seconds) to simulate exposures similar to those used during cyanide fishing.

The conversion rate of CN⁻ to SCN⁻ facilitated by the enzyme rhodanese is not simply a function of enzyme kinetics (Leduc 1984). The conversion rate appears to be limited by the availability of sulfur present in the fish. Likewise, the metabolism and excretion of SCN⁻ from the fish may be related more to osmoregulation than to temperature-mediated enzyme kinetics. Freshwater fish have a higher blood ion concentration (hyper-osmotic) in relation to the surrounding water; while marine fish have a lower blood ion concentration (hypo-osmotic) in relation to seawater. Hence, freshwater fish have a high rate of urinary excretion and marine fish have a low rate of urinary excretion; which helps the fish to maintain osmotic equilibrium with surrounding aquatic environments (Smith 1982).

Since the physiology of freshwater and marine fish is drastically different with regard to the maintenance of osmotic balance (Smith 1982), it stands to reason that the regulation of plasma anions may also differ. It is believed that marine fish retain SCN⁻ for a longer

time period than freshwater fish because of the lower rate of urinary excretion. Hence, the interpretation of results from cyanide exposure studies conducted with freshwater fish to the toxicity and kinetics of cyanide in marine fish should be conducted with caution. Scientific studies are needed concerning the physiology and pharmaco-kinetics of CN⁻ and SCN⁻ with marine fish.

The committee agreed that there was an urgent need for research to determine the pharmaco-kinetics of cyanide and its metabolites in MAF. Without this knowledge, it is difficult to predict whether or not various cyanide testing methods can be applied associated with the chain-of-custody from collectors to export companies situated the Philippines, Indonesia, and Vietnam to importers, wholesalers, and retailers situated in importing countries like the U.S.A.

Field Test In Exporting Countries

In response to threats from terrorism, a number of field tests for cyanide have been developed and evaluated for the US-Environmental Protection Agency (US-EPA) by Battelle. Most of these are either low-cost colorimetric kits (dependent on color changes) or based on ion selective electrodes (ISE) linked to portable ISE meters. These kits mostly detect cyanide in water. One colorimetric test kit discussed by Dr. Rubec is sold by Industrial Test Systems (ITS) and is called the Cyanide ReagentStrip™ Test Kit (Batelle 2005). Thermo-Fisher markets the Thermo Orion 9606 portable ISE equipment. There are other companies with similar products that also were evaluated by Batelle for US-EPA over the past five years.

Mr. Gil Adora (Deputy Director of BFAR within the Philippines Department of Agriculture) and Mr. Agus Dermawan (Deputy Director of the Division of Small Islands

and Marine Parks within the Indonesian Ministry of Marine Affairs and Fisheries) both expressed, in their presentations at the Cyanide Detection Workshop, the need for a rapid, easy to use field test to support law enforcement efforts. The available kits and/or meters generally measure CN^- in solution. These kits may be useful to detect cyanide ion in solution (such as in seized cyanide squirt bottles) or to detect cyanide after suspected tablets seized from collectors/fishers are dissolved in water (fresh or saltwater). The fishers/collectors use either sodium cyanide (NaCN) or potassium cyanide (KCN) for cyanide fishing. Sodium cyanide is mostly used to capture fish by fishers in the Philippines; while potassium cyanide is more commonly employed for cyanide fishing in Indonesia. The committee agreed that portable test kits designed for measuring cyanide in solution are unlikely to be effective in detecting cyanide bound in the tissues of MAF.

Soudararajan Procedure

Another suggested method for testing fish samples for the presence of cyanide was developed by Dr. Rengarajan Soundararajan in 1990 (Rubec and Soundararajan 1991). The method involves the extraction of cyanide from blended fish tissues using concentrated sodium hydroxide (NaOH). The method is appealing since it is relatively simple and quick to conduct. The cyanide ion is released from the tissues into sodium hydroxide (NaOH). The high pH (pH 12-13) of the solution prevents the cyanide from being lost to the atmosphere. Cyanide concentrations can then be measured with an ion-selective electrode (ISE).

Five fish species obtained from Indonesia tested in the U.S.A. had cyanide ion concentrations ranging from 5.8 to 23 mg/kg (ppm) (Rubec and Soundararajan 1991). A Clown Triggerfish obtained from the Philippines exhibited a

cyanide ion measurement of 1120 mg/kg. No cyanide was detected in two Flame Angelfish obtained from the Marshall Islands, for two French Angelfish from the Caribbean, and two species of surgeonfish obtained from Hawaii. Hence, cyanide was detected in fish from countries known to have collectors using cyanide and no cyanide was detected in fish obtained from countries where cyanide is not used for collecting marine aquarium fish.

The International Marinelife Alliance (IMA) evaluated the technique in 1991, but abandoned it after it was found to give anomalously high cyanide readings. It was suspected that the anomalously high readings found by the IMA might have occurred because of false-positive readings caused by sulfide interference with the ISE electrode.

Research by Aquarium Systems was conducted with a small grant obtained by IMA from the Columbus Zoo (Frakes and Studt 1996). The basic procedure was to expose the fish to known concentration of cyanide ion, kill the fish, then puree the samples in 5 Molar (M) NaOH . The NaOH volume was calculated to yield a 10% by weight fish slurry. This slurry was allowed to settle and a clear aliquot was diluted with distilled water to produce another 10% by weight dilution. Cyanide ion (CN^-) concentrations in the final solution, 1% fish tissue, and approximately 0.5M- NaOH were measured in millivolts (mV) recorded with an Orion CN^- ISE linked to an Orion ISE meter.

These readings were compared with a semi-log plot (calibration) produced with known cyanide concentrations of 0.1, 1.0, and 10 mg/L levels with mV readings recorded as each level (Frakes and Studt 1996). Lead carbonate was added to the supernatant solution to precipitate sulfides from solution. The first trial compared readings with Atlantic

Blennies, which were net-caught and exposed to cyanide. Both the test and the control fish (not exposed to cyanide) were found to exhibit cyanide levels of about 300 mg/L (ppm). Hence, the addition of lead carbonate to the solution prior to ISE testing did not eliminate the anomalously high readings.

A second experiment evaluated whether the high readings might be due to iodide interference with the ISE. Again, the experiment was inconclusive. In a third experiment, test fish (4 Caribbean Blue Chromis, 1 Atlantic Wrasse, and 3 Goldfish) were exposed to cyanide concentrations ranging from 1.3 to 3.9 ppm CN⁻ for times ranging from one minute (for the 4 chromis) to 14 minutes (for a goldfish). Readings for control fish (1 wrasse and 2 goldfish) not exposed to cyanide produced higher mV readings than fish exposed to cyanide.

Recent analyses by Ms. Benita Manipula using the Soundararajan procedure and cyanide measurements obtained using an Industrial Test Systems (ITS) colorimetric Cyanide Reagent StripTM (Cyanide Test Kit 484003) have also experienced difficulty in obtaining reliable measurements of cyanide concentrations in comparison to known concentrations of cyanide ion in sodium hydroxide solution (lacking slurry). The problem does not appear to be related to the reliability of the ITS cyanide test strips, since they have been demonstrated to be sensitive (down to 0.02 mg/L) and reliable for measuring cyanide ion concentrations in water (Battelle 2005).

The most likely explanation for these results is that organics in the digested tissue solutions containing sodium hydroxide produced the anomalous readings both with the ISE and the ITS test strips. Dr. Frant suggested that these could be volatile mercaptans from

sulfur-containing proteins. Further research is needed to see whether it is possible to measure cyanide ion concentrations in solutions obtained from marine fish using the Soundararajan tissue digestion method. It does not appear likely that the Soundararajan procedure can be applied as a field test (Rubec and Soundararajan 1991, Rubec and Manipula 2008).

Cyantesmo Screening Test

The workshop committee agreed that it might be possible to digest fish samples in sulfuric acid in a closed container to release hydrogen cyanide (HCN) from the digested sample into the atmosphere of the container. Test strips mounted in the container (such as near the lid of a jar) might be able to provide a colorimetric indication for the presence of cyanide ion. This approach would need further research and field evaluations. It is likely to only work near the point of collection in the exporting countries; where cyanide ion is most likely to be present in the fish at higher concentrations.

Actually, there are several testing protocols based on this idea. The American Society of Testing and Materials (ASTM) published a screening method (D-5049) for screening cyanides in waste (ASTM 1990). The protocol involves adding 5 grams of organic sample to a sealed vial also containing concentrated sulfuric acid. Hydrogen cyanide released from the sample causes a color change with Cyantesmo paper suspended over the sample.

A similar screening protocol (Figure 1) is used by the U.S. Food and Drug Administration (FDA) to analyze various foodstuffs for the presence of cyanide-including meat and vegetables (Flurer et al. 2005). It has been used to detect cyanide with tuna samples (both frozen and canned) spiked with cyanide.

The Cyantesmo paper changes color from light green to dark blue depending on the concentration of hydrogen cyanide liberated.

Several papers have been published using Cyantesmo paper. Flinger et al. (1992) used it as a screening tool to detect the presence of cyanide liberated from blood samples. Relia et al. (2004) evaluated the method with concentrations of cyanide ion ranging from 0.25 to 30 mg/L (ppm). The test strips demonstrated incrementally increasing deep blue color changes over a progressively longer portion of the test strip in less than five minutes for each concentration of cyanide including 1, 3, 10, and 30 mg/L. The concentrations of 0.25, 0.5, and 0.75 mg/L required more than two hours to begin demonstration of a color change.

It is recommended that the FDA protocol be evaluated as a screening tool for the detection of cyanide in fish tissue samples near the

point of collection in exporting countries. The procedure will require the use of regional testing laboratories. It has the advantage that many samples can be analyzed at low cost.

BioSensors

Mak et al. (2005a) presented a summary of various papers that have developed biosensors for detecting cyanide ion. Most of the methods are dependent on measuring chemical reaction products based on enzymatic reactions (e.g., formate production from action of cyanide hydratase, cytochrome oxidase inhibition, peroxidase inhibition, rhodanese and sulphite oxidase reactions). There is also a microbial sensor, and the measurement of oxygen uptake by bacteria. There are efforts being made to link the enzymatic reactions to potentiometers to measure the enzymatic reaction as changes in electrical potentials. The idea is to create a microprobe (containing the enzymes) that would be inserted into the blood to give a reading based on a change in

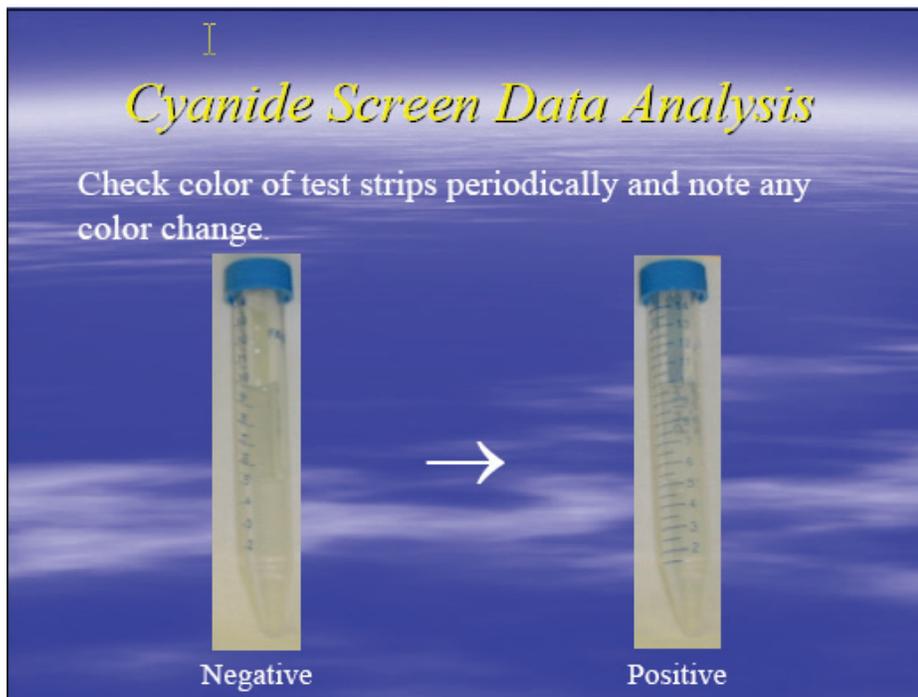


Figure 1. Sealed test tubes used by the U.S. Food and Drug Administration to detect the presence of cyanide in foodstuffs using Cyantesmo paper suspended above an acidified sample. Image provided by Dr. Fred Fricke, USFDA.

electrical potential (similar to an ISE reading). Literature searches by Dr. Rubec and Dr. Frant indicate that no company is presently marketing a microprobe based on biosensors. A potentiometric biosensor is reported to be under development involving the use of an EIS (Electrode-Insulator-Semiconductor) as a microprobe (Kuegsen et al. 2004, Turek et al. 2007).

Research presented by Mak et al. (2005c) suggests that CN^- in solution associated with blended tissue samples (Figure 2, page 133) declined to zero in about one hour. This may be because the cyanide, present in solution was taken up by the tissues in the homogenate. It should be noted that the above experiment by Mak et al. (2005c) does not represent a depuration rate, since they did not demonstrate that cyanide was excreted from the fish in less than one hour.

A study by Bellwood (1981) using radioactive tracers indicated that the cyanide in solution was taken up across the gills and the stomach of the fish. The cyanide in the blood was rapidly taken up by the fish's organs; including the liver, kidney, heart, spleen, and brain. Hence, the CN^- may only be detectable in the blood of marine fish for a few hours using any of the biosensor methods described above. This needs further study. Radioactive tracers may provide a rapid method for determining depuration rates of cyanide and its metabolites in MAF.

Use of ISE Electrode and ISE Meter

Provided that the half life of cyanide ion in the fish is not too short, it should be possible to measure cyanide ion extracted from digested fish tissue samples in a laboratory using the ASTM ISE method (the method used by the BFAR/IMA labs) from 5 to 14 or more days after the fish were captured using sodium cyanide or potassium cyanide. The

fact that the International Marinelife Alliance (IMA) was able to do this, with over 48,000 specimen samples in six BFAR/IMA CDT laboratories in the Philippines between 1993 and 2001 (Rubec et al. 2003), suggests that the half-life in marine fish is longer than the half-life of CN^- reported for humans.

First it should be noted that the IMA did not develop the ISE method for determination of cyanide ion concentrations in solution. The IMA adopted the Standard Operating Procedure (SOP) published in "*Standard Methods For The Examination of Water and Wastewater*", 18th Edition, published by the American Public Health Association (APHA), American Water Works Association (AWWA), and the Water Pollution Control Federation (WPCF) (APHA-AWWA-WPCF 1992) and in the "*1997 Annual Book Of ASTM Standards*" Vol. 11.02, (D2036-91) published by the American Society of Testing and Materials (ASTM 1997). It is also the method used by the US-Environmental Protection Agency (US-EPA 1983). It was developed by the ASTM in the early 1980s and has been repeatedly published by these agencies.

The method uses reflux-distillation apparatus into which chemicals are added to deal with interfering substances (Figure 2). Sulfamic acid is added to reduce interference from nitrates and/or nitrites. Magnesium chloride is added as a catalyst. Concentrated sulfuric acid is added and the solution (acid, other chemicals, and blended tissue sample) heated (using a heating mantle under the flask) for about an hour to digest the fish tissue. Hydrogen cyanide (HCN) gas passes through the reflux condenser and CN^- is captured in an absorption tube containing concentrated sodium hydroxide solution (Manipula et al. 2001b). Lead carbonate added to the absorption tube is used to eliminate sulfide interference. The CN^- concentration is then

measured using an ISE (Figure 3) linked to a pH/ISE meter (Manipula et al. 2001c).

The ISE method for determination of CN^- concentrations has been evaluated through two separate round-robin comparisons by the ASTM and by the APHA (ASTM 1987, 1997, APHA-AWWA-WPCF 1998). Both the ASTM round robin and the APHA round robin evaluations were based on six operators in five laboratories (ASTM 1987, 1997). But, the concentrations tested with water samples and the sample sizes evaluated were different.

With the ASTM (1987) round-robin evaluation, samples of CN^- in various water matrices were sent as unknowns to five laboratories for analyses. The testing done on samples with a known concentration of 0.03 mg/L were determined to have an average concentration of 0.029 ppm (ASTM 1987, 1997). The limit of detection must be well below this level. In fact, Elsholz et.al. (1990) found with a five minute time response that a CN^- concentration of 0.003 mg/L (ppm) was detectable for two different



Figure 2. Distillation apparatus used by IMA chemists to digest tissue samples in order to liberate hydrogen cyanide gas, that is then captured as cyanide ion in an absorber tube containing sodium hydroxide.

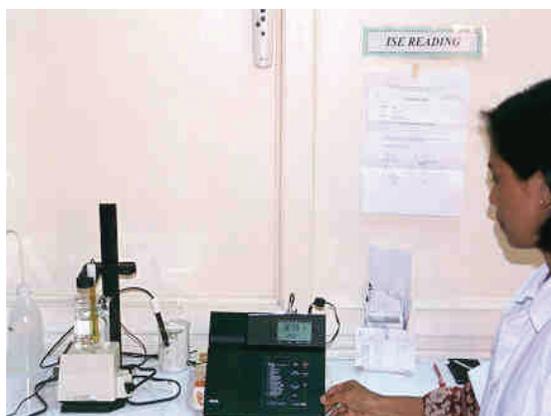


Figure 3. Ion Selective Electrodes (ISE) linked to a Thermo-Orion pH/ISE meter used by the IMA to measure cyanide ion concentrations in sodium hydroxide after reflux distillation.

cyanide ISEs. Hence, CN^- is detectable below 0.03 mg/L, but the concentration may be difficult to determine; since the calibration on a semi-log plot deviates from a straight-line relationship, determined between known CN^- concentrations and the electric potential readings obtained using the ISE apparatus.

There is no doubt by any of the organizations (APHA, ASTM, US-EPA, USGS) that have endorsed this method, that the ASTM ISE method is a reliable method that can be used to determine CN^- concentrations in solution (Ghosh et al. 2006). There are some differences in the methods that have been separately published by these organizations, but they are minor.

The methods published by these agencies involve an acid digestion and distillation of samples, with addition of chemicals to help remove interfering substances (Gosh et al. 2006). Cyanide ion can then be measured in solution using a variety of methods (including but not limited to colorimetric, ISE, HPLC, and mass spectrometric methods).

The International Marinelife Alliance (IMA) conducted cyanide detection testing (CDT)

in the Philippines under contract with BFAR from 1993 to 2001. The ISE method, utilized by the IMA has been criticized by several groups that attempted to repeat the CDT procedures used by IMA (Holthus 1999, Mak 2003, Mak et al. 2005b). This led some people to question whether the ISE method utilized by IMA and presently by BFAR chemists is reliable.

In 1999, the Marine Aquarium Council (MAC) convened a panel of eight scientists to review the SOP manual utilized by the IMA (Manipula et al. 1995). The SOP is essentially the ASTM ISE method discussed above. The panel members noted that the SOP did not contain quality assurance-quality control (QA/QC) procedures (Holthus 1999). The IMA submitted a formal response to this report (IMA-Philippines 1999). The IMA noted that QA/QC procedures were being routinely applied by the six BFAR/IMA laboratories and agreed to incorporate them into a revised SOP. This was done and four new SOP manuals were produced (Alban et al. 2001, Manipula et al. 2001a, 2001b, 2001c).

The main disagreement by IMA (IMA-Philippines 1999) with the MAC report (Holthus 1999) pertains to analyses conducted by an unspecified chemist contracted by MAC to repeat the ISE method. The consultant changed the test apparatus (substituted a 50 ml mini-distillation flask) and made other changes to the procedure. The main criticism by IMA was that the consultant overheated the distillation flask and infused air into the reflux condenser at too high a rate resulting in low percent recoveries of cyanide ion in five experiments involving marine aquarium fish. Tables 2, 3, 4, and 5 indicate percent recoveries of cyanide ion ranging from 31-77% (Holthus 1999). If the ASTM (1997) procedure was correctly conducted one should expect recoveries of CN^- near 100%. The published

procedure states "Equivalent apparatus is acceptable provided cyanide recoveries of 100 ± 4% are documented" (ASTM 1997). Hence, the low percent recoveries of CN^- after the digestion and distillation of cyanide spiked tissue samples reported (Holthus 1999) brings into serious question the validity of the consultant's experimental results (IMA-Philippines 1999). The IMA noted that it routinely had percent recoveries of cyanide greater than 90%. Hence, the low percent recoveries experienced by the consultant were not a problem for IMA chemists who correctly applied the ISE methods described in the SOP.

The other main concern (IMA-Philippines 1999) was that the consultant claimed the levels of cyanide measured with the ISE were below detectable limits (BDL) (Holthus 1999). In three experiments (Tables 3, 4 and 5) cyanide levels recovered from spiked samples ranged from 0.0001 to 0.0005 mg/g. The consultant claimed that the ISE method was invalid because the test results were BDL. The IMA pointed out that the consultant was wrong because the concentrations should have been reported in parts per million wet weight, which is expressed as mg/kg. The results ranged from 0.1 to 0.5 mg/kg (ppm). Hence, the consultant's results were well within the range of detection by the ISE apparatus and were not below detectable limits.

The MAC also contracted Dr. Reinhard Renneberg at the Hong Kong University of Science and Technology to review cyanide detection methods and develop a new cyanide detection method. A graduate student in his laboratory, Karen Mak attempted to repeat the ISE method previously used by the IMA (Mak 2003). Unfortunately, the manual she followed was not the correct SOP manual utilized by the BFAR/IMA laboratories (Manipula 1995). She used a handout (IMA-

Philippines 2000) obtained at a Live Food Fish Trade meeting held in Hong Kong in 2000 that only partially described the actual SOP used by IMA. No attempt was made by either Ms. Mak or Dr. Renneberg to contact IMA to obtain the correct SOP manual.

The assertion by Mak et al (2005b) that “The reflux distillation method together with an ISE was not sensitive enough for the determination of cyanide traces in post-cyanide exposed fish” is incorrect (Rubec 2007). Since, this was associated with a discussion of the methods used by the IMA, the implication was that the IMA could not reliably measure cyanide concentrations below 0.26 mg/L. However, Mak (2003) did not use the same ISE equipment used by the IMA. She used a Pheonix ISE electrode (Cat No. CN01503); connected to a Jenway pH/mV meter (model 3305); which has a range of detection on a linear semi-log plot of 0.26 to 260 ppm (mg/L). The IMA used a Thermo-Orion ISE electrode (#9406BN) that can reliably detect cyanide concentrations down to 0.03 mg/L on a linear semi-log plot (ASTM 1997). Likewise, the pH/ISE meter used by Mak (2003) differs from that used by the BFAR/IMA laboratories (Thermo-Orion Model 920A) (Manipula 1995, Manipula et al.2001c).

The Thermo Orion ISE equipment (ISE electrodes and meter) is capable of detecting cyanide ion in solution with a straight-line calibration on a semi-log plot down to 0.02 mg/L (2 ppb) (Frant et al. 1972; Orion Research Inc. 1975, 1997; Sekerka and Lechner 1976). This was routinely done with daily calibrations conducted by the IMA laboratories using the Thermo Orion ISE equipment (Manipula et al. 2001c). This is similar to round-robin findings by the ASTM (1987).

Mak et al. (2005b) reported that the lower range of detection for cyanide ion using a colorimetric method was 0.026 ppm. Hence, they claimed that the colorimetric method had a higher sensitivity than the ISE method (actually both have about the same lower limit of detection). They claimed that the cyanide reflux distillation method together with the ISE was not sensitive enough for the determination of traces in post-cyanide exposed fish. A series of QA/QC experiments were conducted that are irrelevant considering that the wrong ISE equipment was utilized (Mak 2003). Mak et al. (2005b) mistakenly concluded: “The cyanide distillation method combined with the ISE described in the SOP manual and employed by the IMA laboratories requires considerable modification and elaboration. A reliable and ultra sensitive system for cyanide detection in fish is urgently needed.” It should also be noted that the lower limit of detection for the biosensor method that Mak et al. (2005c) developed is 0.0286 ppm (mg/L), which is about the same as the Thermo Orion ISE method on a linear calibration (Sekerka and Lechner 1976). Hence, it is not more sensitive than the ISE method (ASTM 1997).

The present authors, including Dr. Kobelski and Dr. Logue, believe that the ISE method associated with the SOP manual used by the IMA (Manipula 1995) and by the various agencies that also use the method (including APHA, ASTM, US-EPA and BFAR) is scientifically reliable, although this has not been demonstrated with fish tissue in controlled experiments. This is also the opinion of Dr. Ellen Gonter who helped develop the ISE method for ASTM, Dr. Martin Frant of Thermo Orion who developed the cyanide ISE electrode, and Dr. George Dixon, an expert concerning cyanide physiology in fish and Vice-Dean of Research at the University of Waterloo, Canada (all three provided letters

endorsing the IMA's use of the ISE method after the flawed MAC review).

There was no need to develop a new method (MAC 2004, Mak 2003, Mak et al. 2005b), since the SOP using ISE presently being applied by BFAR (Manipula 1995, Manipula et al. 2001b, 2001c) is reliable and has international acceptance. Any new method would need to go through extensive evaluations and round-robin comparisons before it could be accepted by the scientific community and by agencies regulating the marine aquarium trade.

Dr. Kobelski near the end of the CDT Workshop suggested that round-robin comparisons needed to be conducted involving the ISE method and that comparisons needed to be made with an independent cyanide detection method. He suggested the use Gas Chromatography linked to Mass Spectrometric analyses (GC-MS), since this is the method used by the CDC (Kage et al. 1996, Dumas et al. 2005, Murphy et al. 2006) and has recently been accepted by the US-EPA for the analysis of cyanide in drinking water. A GC-MS apparatus used in Texas to measure cyanide is depicted (Figure

4). This would require that fish be dosed with cyanide and frozen samples be sent to two or three separate laboratories. He suggested that a statistically significant number of samples should be analyzed by the ISE method. This could be time consuming and expensive, if done by laboratories in the U.S.A.

According to Dr. Kobeski there are 46 laboratories in the CDC network, which are qualified for trace analysis of cyanide in clinical samples. The Michigan Department of Community Health has been conducting fish exposure studies to detect cyanide using GC-MS. Preliminary results from the Michigan laboratory found a background level of cyanide in freshwater fish in the 0.01 mg/L (10 ppb) range. The Michigan laboratory has cross-validated the headspace GC-MS method with the distillation ISE method for the US-EPA using cyanide in solution.

Dr. Logue suggested that there may be background levels of cyanide in MAF. It is possible that the cyanide concentrations detected in the fish tested by the BFAR/IMA laboratories were background levels of cyanide rather than cyanide resulting from cyanide fishing. Ms. Manipula (Chief Chemist for the BFAR/IMA laboratories) pointed out that the IMA had tested MAF caught with nets and found no detectable level of CN^- (zero) present in the fish. Hence, she believes that the cyanide being detected resulted from cyanide fishing. Some testing needs to be done to verify that MAF do not have background levels of cyanide, or that the background concentration is below the detectable limit (BDL) determined from the linear calibration for the ISE method.

Dr. Kobelski agreed that the ISE method was probably reliable (Email: February 21, 2008). "I have repeatedly said that I believe that the ISE method is probably valid – my failure to



Figure 4. A gas chromatography-mass spectrometry (GC-MS) apparatus used to determine cyanide concentrations. Image provided by Dr. David Klein, Texas Department of State Health Services.

review all the information is one reason I say probably – I find no flaws with the analysis technique. I do have concerns about the use of sulfuric acid possibly oxidizing thiocyanate to cyanide based on some work I did 20+ years ago. This is not likely to be an issue with drinking water but with biological samples, it is a concern.”

There is almost nothing in the published scientific literature concerning the use of the ISE method for detection of cyanide associated with the digestion and distillation of fish tissue samples (Dzombak et al. 2006). Consequently, Dr. Kobelski noted the following concerns: 1) the possible oxidation of the cyanide by sulfuric acid in the digestion flask; 2) the lack of demonstration of adequate recovery in controlled experiments using spiked tissue samples; 3) that unexposed MAF might have background levels; 4) that the kinetics of cyanide might be too fast for cyanide to be detectable at points of export or import. Since there is no published data available, he proposed that research should be conducted to evaluate these questions.

Need for a Field Test For Ports of Entry In USA

The U.S. Fish and Wildlife Service (USFWS) agents present at the Cyanide Detection Workshop expressed the need for a reliable, rapid, easy to use field test that could be used to detect cyanide or its byproducts at US ports of entry such as Los Angeles, San Francisco, Miami, and New York (where most marine ornamental fishes are imported). There are a number of low-cost test kits that can measure CN^- either using colorimetric methods or using ion selective electrodes (ISE) linked to portable ISE meters.

The authors believe that because the CN^- is metabolized to thiocyanate anion (by the enzyme rhodanese); it is unlikely that cyanide

ion would be detectable using the ASTM ISE method in importing countries like the USA, because of the long time delay before fish reach U.S. points of entry (by air). However, Dr. Kobelski felt that if the kinetics are slow enough for cyanide to be retained in the fish until the time of export, that it might also be possible to detect the presence of cyanide using the ISE method at points of import to the U.S.A. Theoretically it should be possible to measure SCN^- in MAF because it would be retained for a longer time period. The advantage of measuring thiocyanate is that appreciable concentrations of SCN^- may be found following exposure and it has a longer half-life than CN^- (Logue and Hinkens 2008).

Measuring Thiocyanate in Blood

Two research grants were obtained by Dr. Roman Lanno at Ohio State University (OSU) to determine the feasibility of measuring SCN^- in the blood of MAF after they had been imported into the U.S.A. (Lanno et al. 2002a, Lanno et al. 2002b). The objective of the proposed research (Lanno et al. 2002a, 2002b) was to assess whether plasma thiocyanate (SCN^-) could be used as a biomarker of cyanide exposure in marine aquarium fishes captured using the cyanide squirt bottle technique. The objective of the study was to be met by conducting three experiments: i) Validate and refine an existing high pressure liquid chromatography (HPLC) technique for the determination of SCN^- in the plasma of marine aquarium fish, ii) Determine the pharmacokinetics of SCN^- in a marine aquarium fish species exposed to sublethal levels of waterborne cyanide, and; iii) Determine the toxicokinetics of SCN^- in the plasma of a marine aquarium fish species exposed to a pulse dose of cyanide that simulated exposure during actual collection on a coral reef.

The intended products from the research at OSU were to include: i) a detailed manual documenting methods for monitoring SCN^- in fish tissues that can be used to enforce regulations in importing and exporting countries, ii) Scientific publications describing the pharmacokinetics (uptake and depuration) of SCN^- in MAF, outlining the bounds and limitations of the technique.

A graduate student (Julie Koontz) commenced the research in 2003 under the direction of Dr. Lanno. She was able to measure SCN^- concentrations in the blood of several MAF species (*Pseudanthias pleurotaenia* and *Pseudanthias squamipinnis*) using an HPLC method developed by Chen and Yang (1996). This HPLC method is capable of detecting thiocyanate in blood plasma at low levels (0.5 – 1 nmol mL⁻¹). It requires derivatization of thiocyanate anion with 3-bromomethyl-7-methoxy-1,4-benzoxazin-1-one, then separation by a reversed-phase C18 column. Quantification is by HPLC with fluorimetric detection.

Koontz (pers. comm. 2005) experienced difficulty in obtaining enough blood from the MAF, because of their small size. The original method, as described in Chen and Yang (1996), requires 0.5 mL of blood plasma. It may be possible to use less plasma by evaporating the entire 10 mL plasma eluate (rather than a 1.0 mL aliquot) to increase the quantity of thiocyanate ion present. The same change could also increase the sensitivity of the test for the 0.5 mL plasma volume and possibly extend the period after exposure that thiocyanate is detectable.

For personal reasons, Ms. Koontz decided to drop out of the graduate research program at OSU. Hence, the pharmacokinetic research of SCN^- dynamics in MAF was not completed.

Other Methods for Measuring Thiocyanate

There are colorimetric tests for cyanide ion and the thiocyanate anion that could be applied (APHA-AWWA-WPCF 1992, ASTM 1997). It might be possible to measure the presence of SCN^- in blood samples taken from fish using colorimetric methods. A spot test for these ions is published by the APHA-AWWA-WPCF (1998). Provided there are no interfering substances in the sample, the presence of CN^- can be determined by colorimetric methods (e.g., chloramine T, pyridine barbituric acid, phenolphthalein reagents). The CN^- can be masked using formaldehyde and the sample retested. This makes the spot test specific for SCN^- . Whether this approach would work with blood samples is unknown. More research is necessary.

It may be possible to measure SCN^- levels in MAF after their tissues are dissolved in nitric acid and sulfuric acid. More acidic solutions are required to liberate SCN^- from tissues than are required to liberate CN^- . There is an ISE sold by Thermo-Fisher that can measure thiocyanate anion concentrations, after the tissues are digested. There are many colorimetric methods for SCN^- and it is likely that HPLC or ion chromatography could be used. CDC has a liquid chromatography-mass spectrometric (LC-MS/MS) method for the analysis of SCN^- in both blood and urine for human clinical samples. It is unlikely that the thiocyanate ion can be distilled (like the ASTM ISE method used for detection of cyanide ion). More research is needed to determine whether a test for thiocyanate in MAF is feasible.

Dr. Kobelski suggested that measuring thiocyanate in the blood of MAF was not feasible on a routine basis due to the low volume of blood in small ornamental fish. Whatever test is applied in the USA will

probably start with fish tissue or tissue homogenates.

Dr. Logue pointed out, based on mammalian research, that thiocyanate can be produced from several metabolic pathways (Logue and Hinkens 2008). “However, thiocyanate is naturally found in biological fluids, and while this is a condition of all cyanide metabolites, thiocyanate levels are normally quite high and can be inconsistent. Large variation in background thiocyanate concentrations (in mammals) make it difficult to determine low level cyanide exposure....Large and variable concentrations (of thiocyanate) may indicate that thiocyanate is involved in a number of biological processes in addition to cyanide metabolism.” Hence, even if thiocyanate could be detected in MAF being imported, it might not be possible to assert in court that the presence of thiocyanate detected in the fish was absolute proof that the fish were caught using cyanide in the country of origin.

Based on the previous discussion, it is unlikely that a rapid field test for thiocyanate can be applied. Analyses of frozen samples sent to a laboratory (such as the USFWS laboratory in Oregon) will probably be necessary. From a cost perspective, measuring thiocyanate may be much less costly than the ATCA method (discussed below).

ATCA Test

The committee agreed that from a theoretical standpoint it may be better to use a biomarker such as ATCA (Lindquist et al. 2005, Logue et al. 2005, Logue et al. 2007, Logue and Hinkens 2008), especially if the US-based test results will be used for prosecution of those in the aquarium trade. “A minor metabolic pathway is the conversion of cyanide ion to 2-amino-2thiazoline-4-carboxylic acid (ATCA). Another by product (tautomeric form) is

2-iminothiazolidine-4-carboxylic acid (ITCA). The production of ATCA may predominate when sulfur donors become depleted or in tissues where rhodanese is sparse. ... ATCA may be used as an alternative to thiocyanate for determination of cyanide exposure. An advantage of using ATCA is that it is stable in biological samples for months at freezing and ambient temperatures and therefore, may be a lasting signature for cyanide exposure. However, relatively few techniques have been described to analyze ATCA from biological matrices and relatively few studies have been conducted to evaluate the relationship between ATCA and cyanide exposure” (Logue and Hinkens 2008). Nothing is known about the pharmacokinetics of ATCA. A lot of research is needed before it could be applied to support law enforcement.

Since, Dr. Logue has done research on ATCA he may have the equipment. If the USFWS laboratory in Oregon was to adopt this test method, they would need to purchase the equipment. Lundquist et al. (1995) measured ATCA in urine using HPLC. Logue et al. (2005) measured ATCA in blood and urine using GC-MS. It is not clear how ATCA would be liberated from fish tissues prior to analysis. According to Dr. Logue (pers. comm. 2008) the cost of the GC-MS apparatus to measure ATCA with peripheral equipment is about \$125,000. The cost of the equipment and its technical complexity most probably precludes its use for measuring ATCA in exporting countries.

Validation Studies

Dr. Rubec questioned the need for additional studies to evaluate the ASTMISE methodology. There have already been several round-robin studies by the American Society of Testing and Materials (ASTM 1987, summarized ASTM 1997) and by the American Public Health Association (summarized APHA-AWWA-

WPCF 1992, 1998). Both of these studies have included at least 5 separate laboratories and 6 operators but were performed only on aqueous samples, not masticated fish tissue. The concerns about QA/QC, precision, and bias were addressed. The IMA also conducted round-robin comparisons between the six laboratories it ran under contract to BFAR (IMA-Philippines 1999, Rubec et al. 2003). If round-robin testing needs to be conducted, it should be done to validate a U.S.-based test for either thiocyanate or the ATCA method.

It is unlikely that the ASTM ISE method will be applied in the USA. It looks like it will be used in both the Philippines and in Indonesia. An accreditation system tied to CDT analyses from export facilities may give the U.S.A. the means to prove intent. If fish arrive at an import facility along with an Accreditation Certificate, the importers would have to admit that they knew the fish were accredited as being cyanide-free. If a test such as the ATCA proves otherwise, there may be grounds for prosecution of the importer under the Lacey Act. Congressional legislation similar to that proposed by Congressman Ed Case of Hawaii (House Resolution 4928 for 108th Congress in 2004) may be necessary to allow law enforcement officials (from USFWS and/or NOAA) to prosecute importers who knowingly import fish that are proven to contain cyanide. The test that would need to hold up in court would not be the ASTM ISE method. It would be either the thiocyanate test or the ATCA test conducted in the U.S.A. U.S. officials need to investigate the legal ramifications to see whether this is feasible.

Funding

The IMA conducted CDT in six laboratories in the Philippines under contract to BFAR for about \$100,000 per year. The same testing in the U.S.A. would be at least five times more

expensive based on the disparity of salaries between the two countries.

The Philippines, Indonesia, and Vietnam need long-term funding to establish and maintain CDT networks in each country. Unlike the U.S.A., the CDT laboratories in the exporting countries should be used primarily as a deterrent to cyanide fishing. Cyanide testing should be conducted regionally using the cyanide screening method, so that local governments or municipalities can better manage their fisheries. When samples are found positive using the screening method, frozen specimens would be shipped to CDT laboratories established near international airports. The CDT laboratories using the ISE method need to be tied to a licensing system for fishers and MAF collectors, middlemen, and exporters. If fish coming from collectors or fishers situated in municipalities, or from exporters situated near points of export, are found to have cyanide present more than 3 or 4 times, their licenses can be revoked. Prosecution, being expensive, should only be used as a last resort. Prosecutions should probably only be conducted when either the fishermen/collectors, the middlemen, or the exporters are apprehended with cyanide in their possession and the fish test positive for the presence of cyanide.

It should be noted that it becomes more difficult to prove that fishes have been collected with cyanide by the time the fish enter the U.S.A. U.S. agencies such as the U.S. Agency for International Development (USAID) or other agencies, such as the World Bank or the Asian Development Bank, should fund the creation of cyanide detection networks in countries where cyanide use is prevalent. It will be cheaper to conduct most of the CDT in the exporting countries.

The research proposed and the use of an ATCA test to support law enforcement in the U.S.A. can be justified not only on the basis of the aquarium trade (in which cyanide contaminated fish enter the U.S.A), but also from the point of view of counter-terrorism. An accurate, reliable test for either the cyanide ion or thiocyanate anion, and/or a cyanide biomarker like ATCA is needed to protect Americans from attacks by terrorists using cyanide. The costs for the US-based cyanide detection equipment, for the evaluations, research, and trainings are higher than testing done in the exporting countries. However, they can easily be justified based on the terrorism threat.

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Validation Methods for the Determination of Cyanide and Cyanide Metabolites in Coral Reef Fishes

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The consensus of the Working Group was that there exist serious gaps in the understanding of the metabolism and elimination of the cyanide anion and its major metabolites in fish. Without a better understanding of these processes the implementation of an exposure assessment method that would meet legal requirements would be difficult. The recommendation of the Working Group is that a research program be designed to generate the data necessary to determine the availability of cyanide and its metabolites for exposure assessment versus the time involved with the local collection of fish, their transport to a fixed site laboratory prior to export and their arrival at the import destination prior to the implementation of an analysis method at any point in the delivery chain.

Background

In a number of locations both ornamental and live food fish are captured using techniques such as stunning with an aqueous solution containing the cyanide anion (CN⁻). This technique is destructive to the reef, the individual fish and the sustainability of the population and is illegal in Indonesia, the Philippines and Vietnam. To determine if fish are captured using this destructive technique there must be a forensically supportable analysis for cyanide exposure in reef fish implemented in a timely fashion after capture of the fish.

The length of time between capture and delivery of the fish to a capable laboratory can range from five to fourteen days. For a laboratory analysis to be viable the analyte must be stable and at elevated levels in the sample long enough for the sample to be delivered to the laboratory and for the analysis to be conducted. Data from mammals suggests that the cyanide anion is readily metabolized to thiocyanate (SCN⁻) and 2-aminothiazoline-4-carboxylic acid (ATCA), with a half-life of approximately one hour. If metabolism in fish is similar there would not be an elevated level of cyanide in a fish when it reached the laboratory site. For there to be an elevated level of cyanide after five days the half-life would have to be approximately 10 hours, but this toxicokinetics parameter has not been determined.

To determine what analyte can be targeted for forensic analysis the following research needs to be conducted:

- 1) the half-life of cyanide in fish has to be determined and the variation in half-life with respect to different species must be defined,
- 2) the half-life of thiocyanate, the major metabolite of cyanide, in fish must be determined as well as the specie-to-specie variation in half-life

- 3) the metabolism of cyanide to ATCA must be demonstrated and if this is a metabolic pathway for fish the half-life of ATCA must be determined as well as the specie-to specie variation in half-life,
- 4) the background level of cyanide in fish must be determined, if it is above the limit of quantitation (LOQ) of the analysis technique, to define when an elevated level due to cyanide exposure has been encountered.
- 5) The background level of thiocyanate in fish must be determined, to define if an exposure to cyanide will result in a statistically meaningful elevation of background thiocyanate
- 6) The background level of ATCA must be determined, if it is above the LOQ of the analysis technique, to define when an elevated level due to cyanide exposure has been encountered.

To perform this research, validated methods for the determination of cyanide, thiocyanate and ATCA in homogenized fish tissue must be used. These methods must demonstrate adequate sensitivity, specificity, accuracy and precision as determined by the “fit for purpose” concept.

3. Outline of Validation Procedure:

3.1 Method validation is a process by which a laboratory confirms by examination and the provision of objective evidence that the particular requirements for specific use are fulfilled. It serves to demonstrate that:

3.1.1 The method can detect, identify and quantitate an analyte(s):

- In all matrices to be analyzed
- With a demonstrated sensitivity, specificity, accuracy, reproducibility, ruggedness and precision to ensure that results are meaningful and appropriate for decision making by the receiving network.

3.1.2 The method will function reliably for its intended purpose as defined by the network.

3.2 The method developer validates a method by conducting experiments to determine or verify a number of specific performance characteristics that serve to define and quantify method performance.

4. Reference(s):

4.1 Food and Drug Administration, Laboratory Manual, ORA Laboratory Procedure, Volume II, ORA-LAB5.4.5, Methods, Method Verification and Validation

4.2 International ANS/ISO/IEC Guide to the Expression of Uncertainty in Measurement, 1995

4.3 International ANS/ISO/IEC Guide 2 (VIM), 2004

4.4 International ANS/ISO/IEC Guide 8402, Quality Management and Quality Assurance

Vocabulary, 1994

4.5 Foundations of Clinical Research, Applications to Practice, Leslie Gross Portney, Mary Watkins, Appleton & Lange, 1993

4.6 Validation and Peer Review of U.S. Environmental Protection Agency Chemical Methods of Analysis”, Forum on Environmental Measurements, October 14, 2005

4.7 Protocol for the Design, Conduct, and Interpretation of Method-Performance Studies, *Pure and Applied Chemistry*, 67, No. 2, 1995, 331-343.

4.8 Harmonized Guidelines for Single-laboratory Validation of Methods of Analysis, *Pure and Applied Chemistry*, 2002, 74, 835 - 855.”

5. Specific Procedure(s):

5.1 Prior to submitting methods to the network:

5.1.1 The intended use of the method should be defined

5.1.2 The intended use should be aligned with network capabilities

5.1.3 A study plan for method validation should be submitted to and approved by the involved network for review before starting the validation.

5.1.4 A written procedure which includes all the following elements must be prepared:

- Intended use and criteria for use
- Assay principle and safety precautions
- Acceptable sample types, sample collection method, preparation, preservation, storage and transportation conditions
- Description of reagents supplied or directions for preparation and QC
- Description of quality controls to be used.
- Detailed instructions on how to perform the method.
- Detailed instructions on how to interpret and report the result. ,
- Any limitations of the method that are known or suspected
- A summary of the performance characteristics of the method

5.1.5 Written directions (specifications) for the production and QC of the reagents, if provided for the method

5.2 Methods should be validated whenever any of the following occur:

5.2.1 Submission of a new method to the network for inclusion as an official method.

5.2.2 Expansion of the scope of an existing network method to include additional analytes

or new matrices.

5.2.3 Modification of a network method's range beyond validated levels.

5.2.4 Modification of a network method that may alter its performance specifications. This includes: changes to the fundamental science of an existing method, equivalence issues such as substitutions of reagents/apparatus, or changes to some instrumental parameters. Since it is difficult to predict the results of any change, all but the most trivial of changes should be evaluated for effects on method performance.

5.3 Performance specifications required to validate a method.

5.3.1 Performance specifications that should be determined to validate a method will vary depending on the intended use, the type of method being validated, and the degree to which it has previously been validated. All methods submitted must have all the proper controls and the parameters for calibrating and operating the method instrumentation included in the written procedure.

5.3.1.1 Typical validation characteristics which should be considered are the following:
(See Glossary for definitions of these characteristics in Appendix 1)

Characteristics of quantitative methods

- Method uncertainty
- Minimum quantifiable concentration
- Detection limit (See MDL and MDC)
- Applicable analyte concentration range
- Accuracy
- Precision
- Analytical specificity
- Linearity
- Ruggedness/robustness
- Clinical sensitivity
- Clinical specificity

5.3.1.2 Validation tools (Ref ORA-LAB SOP# 5.4.5)

The following tools should be used to demonstrate the ability to meet method specifications of performance:

- Blanks: Use of various types of blanks enables assessment of how much of the result is attributable to the analyte and to other causes.
- Reference materials and certified reference materials with typical interferences expected. Use of known materials can be incorporated to assess the accuracy of the method, as well as for obtaining information on interferences.
- Fortified (spiked) materials and solutions: Recovery determinations can be estimated from fortification or spiking with a known amount of analyte. (Note: Understanding that spiked recovery may not be truly representative of recovery from naturally incurred analytes.)
- Replication: Replicate analyses provide a means of checking for changes in precision in an analytical process which could adversely affect the results.

- Statistics: Statistical techniques are employed to evaluate accuracy, precision, linear range, limits of detection and quantification, and measurement uncertainty.

5.3.1.3 General Validation Protocol Guidance

The following provides guidelines that should be used to determine method performance characteristics:

- Quantitative measurements, e.g. determine limit of quantitation (LOQ), linear response. Minimally need LOD
- Prepare and analyze spiked blanks, matrix samples of known concentration utilizing at least three different concentration levels: low, medium, high based on the intended use of the method. These samples are carried through the complete sample preparation procedure, extraction and analytical steps of a particular method. Matrix effects can also be assessed with these samples. Accuracy and precision are calculated from these results; data will also evaluate robustness of the method resulting from changes in the sample matrix. (Note: Proper certified reference materials and reference standards are used when available.)
- Assure that adequate sample replicates are performed and that results from replicate measurements of each analyte are compared.
- Analyze blanks (reagent and matrix) and compare these results to the reported limit of detection
- Evaluate interferences: spectral, physical, chemical or memory by analyzing samples containing various suspected interferences in the presence of the measurand.

5.3.1.4 Validation of Methods (Original, New or Modified), should include but not be limited to matrix extensions and platform changes.

- In cases where the sample preparation and/or the extraction procedure/analytical method is modified from the existing test procedure and protocol, the new method should demonstrate that the modifications do not adversely affect the precision and accuracy of the data obtained.
- In order to implement the modified method, the standard or existing method is first performed. The modified method is then verified against the original method validation protocol as defined in section 5.3.1.3.
- For original or new methods the authors should pick a validation level that is suitable for their situation as defined in section 5.4.

5.4 Levels of Validation

5.4.1 It is the responsibility of the individual network to determine the level of validation that is acceptable for use on the particular method.

5.4.2 This section references four levels of validations:

Level One: The method is tested in one laboratory for one or more analytes and one or more matrices. The laboratory would select a limited number of key characteristic to evaluate the method performance. (See tables 1, 2, and 3 for specific details for level 1 validation)

Level Two: The method should be validated in a single laboratory. (See tables 1, 2, and 3 for specific details for level 2 validation)

- “Wherever possible and practical a laboratory should use a method of analysis that has had its performance characteristics evaluated through a collaborative trial conforming to an international protocol. Page 844 Pure and Applied Chemistry, 2002, 74”
- “Single-laboratory validation requires the laboratory to select appropriate characteristics for evaluation from the following: applicability, selectivity, calibration, accuracy, precision, range, limit of quantification, limit of detection, sensitivity, and ruggedness. Page 845 Pure and Applied Chemistry, 2002, 74.”

This is similar to the MARLAP tiered project method validation approach for radio analytical methods.

Level Three: The method should be validated by testing the applicable performance characteristics from a single laboratory validation study using two to seven labs with one or more matrices. (See tables 1, 2, and 3 for specific details for level 3 validation)

Level Four: The method is tested using the criteria for a full collaborative study. The study should examine bias, recoveries, applicability, interference, method comparison, calibration procedures and all applicable performance characteristics examined in a single laboratory validation. (See tables 1, 2, and 3 for specific details for level 4 validation)

- For a single type of substance at least 5 materials (test samples) must be used; only when a single level of specification is involved for a single matrix may this minimum required number of materials be reduced to 3. Page 334, Pure and Applied Chemistry, 67, No. 2, 1995.
- At least 8 laboratories should report the results for each matrix; only when it is impossible to obtain this number may the study be conducted with fewer, but with an absolute minimum of 5 laboratories. Page 335, Pure and Applied Chemistry, 67, No. 2, 1995.
- The entire protocol is outlined in the article “Protocol for the Design, Conduct, and Interpretation of Method-Performance Studies, Pure and Applied Chemistry, 67, No. 2, 1995, 331-343.

The Analysis of Cyanide and its Metabolites in Biological Samples

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Cyanide is a toxic chemical that may be introduced to living organisms as a result of both legal and illicit uses of cyanide. Exposure to cyanide can be verified by analyzing cyanide or one of its break-down products from biological samples. This verification is important for medical, law-enforcement, forensic, research, and veterinary purposes. This review will identify common problems associated with the analysis of cyanide and its metabolites, discuss current bioanalytical techniques used for verification of cyanide exposure, and briefly address the metabolism and toxicokinetics of cyanide and its break-down products in biological systems.

Introduction

Cyanide is toxic in humans, animals, and fish and exposure can occur in various ways. Many substances are potential sources of cyanide exposure including edible and non-edible plants, industrial operations, fires, and cigarette smoke. Although the primary natural source of cyanide poisoning is from plants (1-7), other natural sources include volcanoes, bacteria, and fungi (1, 8-13). Man-made sources include malfunctioning catalytic converters, residential and commercial fires involving the burning of plastics, cigarette smoke, as well as illicit uses of cyanide (14). Additionally, hundreds of thousands of tons of cyanide are manufactured annually in the U.S. alone for industrial uses, including chemical syntheses, electroplating, plastics processing, paint manufacturing, gold and silver extraction, tanning, and metallurgy.

Along with many legal industrial uses of cyanide, multiple illegal uses of cyanide exist. Recently, terrorist acts involving cyanide have been the most publicized illicit uses of cyanide. In 1982, cyanide was placed in bottles

of Tylenol in the Chicago area, killing seven people (1). In 1995 in Tokyo, an acid and a cyanide salt were found in several subway restrooms in the weeks following the release of nerve agents (2). Another illegal use of cyanide is the capture of fish for subsequent sale in the live fish trade (15). Cyanide is used at sub-lethal doses to temporarily stun fish, making them easier to catch (16). The practice of using cyanide in this manner has been found in a number of countries, and has an adverse affect on coral reefs (15). The cyanide is toxic to algae that are necessary for coral to survive and also produces many adverse secondary effects, such as killing smaller fish species. With this and other destructive practices used during capture of cyanide stunned fish, irreversible damage to coral reefs has and continues to occur (17).

As industrial and illicit applications of cyanide increase, the need for rapid, sensitive, and specific analytical methods to assess cyanide exposure will be amplified. The goals for this review are to briefly discuss current bioanalytical techniques used for verification

of cyanide exposure and to identify common problems associated with the analysis of cyanide and its metabolites in biological samples. This review does not include methods to test for cyanide in environmental or industrial matrices (e.g., surface waters, minerals, process streams). A number of related reviews have been published (8, 15, 18-22).

the gills or intestine (15). Little is known about the metabolism of cyanide in fish, and more research is necessary to fully understand the similarities and differences of mammals and fish, but considering that cyanide's toxic effects are similar in both mammals and fish, a number of metabolic pathways may also be similar. As the majority of research on cyanide metabolism has been done on

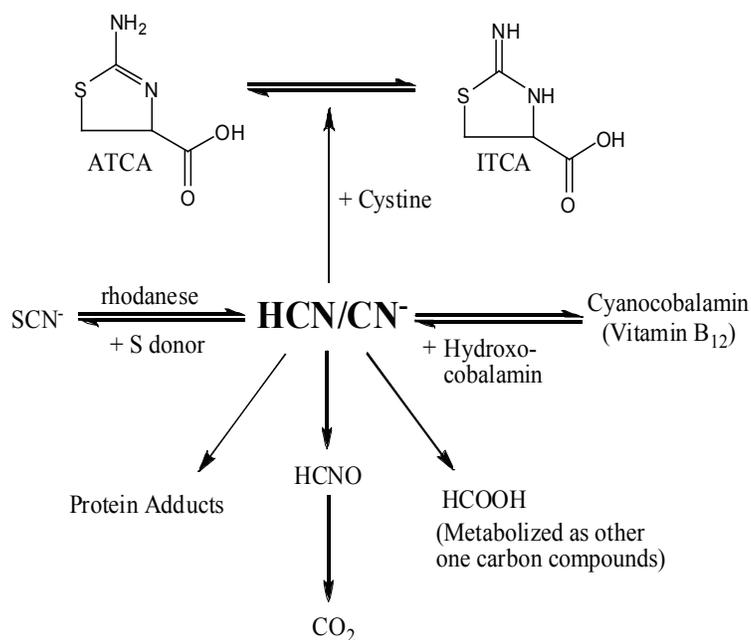


Figure 1. Human metabolism of cyanide.

Cyanide metabolism and toxicokinetics

Although there are other chemical forms of cyanide, it is hydrogen cyanide (HCN) that is the primary toxic agent, regardless of its origin. The toxic effects of cyanide can be traced to interference of aerobic metabolism (18). This occurs when cyanide blocks terminal electron transfer by binding to cytochrome oxidase for both mammals and fish (18, 23). For mammals, cyanide ion (CN^-) is acquired through ingestion while hydrogen cyanide is acquired through inhalation or absorption through the mucous membranes or skin. For fish, cyanide is absorbed through

humans, the following text summarizes cyanide metabolism in humans.

Once absorbed, cyanide is quickly transferred to the blood and metabolized through a number of processes, as shown in Figure 1. The major pathway for cyanide metabolism is conversion of cyanide to thiocyanate (SCN^-) in the presence of a sulfur donor (e.g., thiosulfate) (24). This reaction is catalyzed by the enzyme rhodanese (18, 25, 26). About 80% of the initial cyanide dose is converted to thiocyanate, which is subsequently excreted in the urine. Other minor metabolic

pathways are the conversion of cyanide to 2-amino-2-thiazoline-4-carboxylic acid (ATCA; the tautomeric form of ATCA is 2-iminothiazolidine-4-carboxylic acid – ITCA; Figure 1) in the presence of cystine (18, 26-28), and the reversible reaction of cyanide with hydroxocobalamin to form cyanocobalamin. (Note: Throughout the text, the ATCA/ITCA tautomeric pair will be referred to as ATCA.) The production of ATCA may predominate when sulfur donors become depleted or in tissues where rhodanese is sparse. Other minor pathways for metabolism include the creation of one-carbon metabolites and protein adducts (i.e., reaction of a chemical species with a protein to form a chemical bond that modifies the parent protein) (18, 29). Each metabolite in Figure 1 could potentially be used as a marker for cyanide exposure.

The toxicokinetics and metabolism of an analyte must be considered when an analytical technique is used to determine exposure to the analyte. A main consideration for determining a target for confirmation of exposure is the half-life of the biomarker of interest. Table 1 lists the half-lives of cyanide and thiocyanate for acute exposures of a number of mammalian species. If the half-life of a toxic agent is short, as with cyanide ($t_{1/2} = 0.34-1.28$ hours), it can be difficult to determine exposure by direct analysis of the toxic agent if significant

amounts of time have elapsed. Thiocyanate offers a longer half-life ($t_{1/2} = 4.95-5.8$ hours). The half-lives of ATCA and cyanide-protein adducts are unknown, although for protein-adducts, a half-life similar to that of the parent protein could be expected (e.g., 20-25 days for human serum albumin) assuming the adduct is stable (30, 31). It should be noted that chronic exposure to cyanide increases the apparent half-life of cyanide and thiocyanate (32). This is most likely due to the depletion of sulfur donors over the course of the chronic exposure with subsequent reduction of cyanide transformation by this metabolic pathway. The activity of rhodanese could also be reduced over the course of the chronic exposure, leading to longer half-lives of cyanide.

As seen by the half-lives of cyanide among a number of mammals in Table 1, the toxicokinetics of cyanide is somewhat species dependent. Therefore, although generalities concerning the toxicokinetics and metabolism of cyanide can be made, half-life values cannot be used directly from other species. To more fully understand the relationship of cyanide metabolism and toxicokinetics of sparsely studied species (e.g., most fish species), more research should be undertaken to determine relationships between organisms of interest.

Table 1. Reported half-lives of cyanide and thiocyanate from acute exposures of cyanide.

Cyanide/ Metabolite	Species	$t_{1/2}$ (hr)	Reference
Cyanide	Human	0.34-1.00	(34)
	Rat	0.64	(35)
	Pig	0.54	(35)
	Goat	1.28	(35)
Thiocyanate	Rat	5.80	(35)
	Pig	4.95	(35)
	Goat	13.9	(35)

Special considerations for the analysis of cyanide and its metabolites

Considering the typical half-life of cyanide for acute exposure (Table 1), it may be difficult to determine cyanide concentrations in blood or tissue for long periods of time following exposure. When analyzing cyanide from blood (the preferred method for determination of cyanide exposure in large species), analysis must occur soon after exposure since the concentration of cyanide in the blood decays within minutes to hours. A number of issues limit the direct analysis of cyanide from blood, including rapid cyanide detoxification processes, the difficulty of establishing steady state cyanide levels with time, and the volatility and nucleophilic properties of cyanide. Although limited, direct analysis of cyanide from blood is still useful in that it may be the only biomarker capable of indicating exposure to cyanide within the initial minutes following exposure (33-35).

Although traces of cyanide in urine (36, 37), saliva (25, 38), and expired air (39-42) have been found, direct analysis of cyanide from these matrices is even more limited. It is difficult to assess decay of cyanide concentrations in tissues, because levels of rhodanese are highly variable between organs (18). Considering the highly nucleophilic nature of cyanide ion, free cyanide concentrations are likely to be very low in tissues, although this does not preclude some reservoir of bound cyanide within the tissues. Any cyanide analysis technique would have to consider conversion of bound cyanide to free cyanide prior to analysis.

As an alternative to direct analysis of cyanide, thiocyanate and ATCA may be determined in urine, saliva, tissue, and blood. Cyanide-protein adducts have also been found in human blood proteins. These markers may offer an advantage in half-life (Table 1 for thiocyanate), and correlation of thiocyanate

and ATCA concentrations to cyanide exposure have been examined (43-49). Each of these markers has advantages and disadvantages when considering the detection of cyanide exposure.

The advantages to measuring thiocyanate are that appreciable concentrations may be found shortly following exposure and it has a longer half-life than cyanide (35). However, thiocyanate is naturally found in biological fluids, and while this is a condition of all cyanide metabolites, thiocyanate levels are normally quite high and can be inconsistent (25, 43, 50-55). Large variation in background thiocyanate concentrations makes it difficult to determine low-level cyanide exposure. Also, Ballantyne (56) found that concentrations of thiocyanate in blood varied inconsistently during storage at a number of different temperatures and that analytical recovery of thiocyanate from whole blood was difficult. Large and variable concentration may indicate that thiocyanate is involved in a number of biological processes in addition to cyanide metabolism. Indeed, significant use of thiocyanate by biological processes other than cyanide metabolism has been established (24, 57, 58).

ATCA may also be used as an alternative for determination of cyanide exposure. An advantage to using ATCA is that it is stable in biological samples for months at freezing and ambient temperatures (45, 48). It also has been found that ATCA is not metabolized further (18, 47, 59) and therefore, may be a lasting signature of cyanide exposure. However, relatively few techniques have been described to analyze ATCA from biological matrices (45, 48, 60, 61) and relatively few studies have been performed to evaluate the relationship between ATCA and cyanide exposure (45, 48). With more knowledge of ATCA's behavior with relation to cyanide exposure, ATCA's stability and applicability

to sensitive analytical techniques may prove beneficial.

Recently, cyanide-protein adducts have been discovered (29). If these proteins are stable, they could serve as long-lived markers of cyanide exposure. Disadvantages of this analysis include costly instrumentation, limited research pertaining to the behavior of these adducts, and the difficulty and length of sample preparation. Even considering these disadvantages, cyanide adducts are extremely promising for providing a long-lived biomarker of cyanide exposure, especially if a less complex and less costly method of analysis can be developed.

Multiple factors must be considered when choosing which analyte to target for verification of cyanide exposure. Analysis of cyanide or any one of its metabolites has advantages and disadvantages. Table 2 compares cyanide and its metabolites in terms of some factors important for verification of cyanide exposure.

Species considerations for verification of cyanide exposure

For humans, determination of cyanide exposure has been suggested or attempted from blood (20, 25, 37, 43, 45, 62-97), urine (36, 37, 45, 48, 89, 95, 98), saliva (25, 38, 51, 93, 99), expired air (39, 42), and tissue (post-mortem) (74, 96, 100) samples. Blood may be the most versatile biological sample used to determine exposure to cyanide, because the analysis of cyanide, thiocyanate, ATCA, and cyanide-protein adducts can be performed on blood samples. Saliva, urine, or tissue may be more appropriate depending on the analytical method and other factors, including ease of obtaining the sample. If determination of cyanide exposure is to be done from non-human species, obviously saliva and urine samples will be difficult to obtain. Therefore, blood and tissues are the most likely samples to be analyzed to determine cyanide exposure. The choice of blood or tissue is determined by a number of factors, including the size of the species. For example, most fish species would not have sufficient quantities of blood to analyze, therefore making the analysis of

Table 2. Comparison of cyanide and its metabolites for verification of cyanide exposure.

Cyanide/ Metabolite ^a	Half-lives	Toxicokinetic Data	Storage Stability	Biological Sample ^b	Species ^c
CN	Minutes-hours	Some in a few species	Low	Blood, Urine, Saliva, Tissue, Expired Air, Rumen	Human, Fish, Cow, Mouse, Rat, Guinea Pig, Goat, Horse
SCN	Hours	Limited	Medium	Blood, Urine, Saliva, Tissue, Milk, Gastric Fluid, Cerebrospinal Fluid	Human, Fish, Rat, Mouse, Pig, Goat, Horse
ATCA	Unknown	None	High	Blood, Urine, Feces, Saliva, Tissue	Human, Fish, Rat
CN-protein adducts	Presumably days-months	None	Unknown	Blood	Human

^a CN – cyanide, SCN – thiocyanate, ATCA – 2-amino-2-thiazoline-4-carboxylic acid.

^b Cyanide or the metabolite has been analyzed from this matrix with an analytical method reported in at least one research article or in the authors' laboratories.

^c Cyanide or the metabolite has been analyzed from this species with an analytical method reported in at least one research article or in the authors' laboratories.

tissue samples indispensable. Although tissue may be the only choice for certain species, most published methods for determination of cyanide exposure are for analysis of biological fluids. While some methods developed for biological fluids may fail for tissue analysis, a number of these bioanalytical methods can be slightly modified for analysis of tissues. Another consideration is that rates of cyanide metabolism may be inconsistent between organs because of variable rhodanese and sulfur donor concentrations (18). Therefore, careful selection of tissue, depending on the analyte to be determined, can be extremely important. Also, analytes must be extracted from the tissue for most analytical methods. Therefore, an extra extraction and solid sample processing may be necessary for tissue analysis.

The detection of cyanide and its metabolites in biological samples

The determination of cyanide, thiocyanate, ATCA, and cyanide-protein adducts in biological fluids and tissues, is useful for forensic, clinical, research, law enforcement, and veterinary purposes. Methods of analysis include spectrophotometric or fluorescence methods (37, 41, 43, 44, 51, 60, 61, 67, 76, 77, 82, 87, 89, 99-129), electrochemical methods (90, 130-139), gas chromatography (33, 38, 45, 62, 63, 66, 70, 78, 79, 81, 83, 86, 94, 124, 140-158), and liquid chromatography techniques (37, 46, 48, 69, 80, 84, 88, 89, 93, 95, 97, 98, 133, 159-171). Choosing from the many available types of methods and biomarkers of cyanide exposure, complicated by numerous discrepancies in the literature between these methods makes selection of an analytical method for a specific purpose nontrivial. Factors that will influence the initial choice of which biomarker and analytical technique to use are cellular absorption and detoxification kinetics, sampling and analysis time, sample storage time and conditions,

sample matrix, interferences, sensitivity, available instrumentation and equipment, expertise, and cost. Table 3 describes some differences between the groups of methods listed above in terms of these considerations.

Sample preparation and storage

Careful sample preparation of biological samples containing cyanide or its metabolites is a key element to producing accurate results. (Note: It is always necessary to consider the volatility of HCN when working with samples that may have significant concentrations of cyanide and the dangers that it may pose to laboratory personnel.) A major problem in the analysis of cyanide and thiocyanate is their interconversion, which occurs during sample preparation and storage and leads to inaccurate results. The amount of cyanide within the sample can be altered during storage by up to 66% in 14 days, depending on the storage temperature (20, 33, 77, 172-175). A number of researchers have attempted to address this problem. In preparation of samples for gas chromatographic (GC) analysis, Seto et al. (79) demonstrated that artificial formation of HCN from thiocyanate in blood occurred, and later showed that ascorbic acid prevents artifactual cyanide formation at temperatures below 63°C (175, 176). Sano et al. (88) found that, under the conditions studied, pretreatment of blood samples with water and methanol was successful in preventing artifactual formation of cyanide from thiocyanate. There are a number of methods to help prevent artificial formation of cyanide during storage, and if samples containing cyanide are to be stored before analysis, these methods should be considered (see *Suggested procedures for delayed analysis of biological samples* section). The stability of ATCA in biological samples under a number of storage conditions has been established (45, 48).

Earlier methods of cyanide analysis involved extensive sample preparation in which the sample was acidified (typically with sulfuric or phosphoric acid), and HCN was transferred to alkaline solution by distillation or microdiffusion. This served to concentrate the cyanide and to separate it from potential interferences (33, 43, 70, 77, 82, 105-107, 125, 144, 151). Buffered hydroxocobalamin (67, 177) or methemoglobin (100) solutions have also been used to capture liberated HCN. This pre-treatment method can be used prior to most analytical techniques for the determination of cyanide. For example, this procedure has been used prior to ion-selective electrode (ISE) analysis (15). For GC analysis of HCN, the procedure of liberating HCN by acidification (without capture in solution) is extensively used prior to headspace analysis (33, 70, 94, 144). It should be noted that when using methods that liberate HCN, rubber septa or stoppers can react with gaseous HCN. Therefore, polytetrafluoroethylene septa should be used (64, 151).

Individual pretreatment steps (i.e., derivatization) are generally necessary for detection of cyanide by spectrophotometry or fluorescence. For example, Lundquist and Sörbo (123) used a modification of the König reaction (Figure 2; discussed in more depth in the *Spectrophotometric, luminescence, and atomic absorption methods* section) to spectrophotometrically determine blood cyanide concentrations by high-performance liquid chromatography (HPLC) and 2,3-naphthalenedialdehyde (NDA) and taurine have been effectively used with HPLC-fluorescence or as a stand-alone fluorescence method to produce highly sensitive methods for the determination of cyanide (36, 37, 69, 84, 88, 159). Other derivatization schemes will be discussed with individual analytical methods below.

Thiocyanate sample preparation is normally limited to derivatization that is intended to increase a specific detector's response to the ion. While initial sample preparation is not common, ion exchange columns could be used to separate thiocyanate from biological sample components. Thiocyanate is weakly spectrophotometrically active, but it is normally derivatized with a strong absorber or fluorophore prior to analysis because of its weak absorbance. For example, 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one has been used effectively for the fluorometric determination of SCN by HPLC (163). Others have also used variations of the König reaction to produce stronger spectrophotometric signals (95, 118, 123).

Multiple methods have been proposed for the simultaneous analysis of cyanide and thiocyanate. Methods for simultaneous analysis are generally limited to chromatographic methods and preparation can involve derivatization. For example, Kage et al. (83) used pentafluorobenzyl bromide as the derivatizing agent for simultaneous GC-mass spectrometric (MS) analysis of cyanide and thiocyanate, and Funazo et al. (146) quantitatively methylated cyanide and thiocyanate for GC analysis with a nitrogen-phosphorous detector (NPD). Multiple authors have used other derivatization techniques for GC analysis of cyanide or thiocyanate in biological samples (145, 150, 153, 154).

ATCA has been mainly prepared for analysis using cation exchange solid-phase extraction columns and individual pretreatment steps, depending on the analytical technique. Lundquist et al. (48) and Logue et al. (45) both used cation exchange solid phase extraction columns to separate ATCA from a number of components in biological samples. Lundquist et al. (48) further purified disulfides from samples

by reduction and subsequent separation with another column. Both Bradham et al. (60) and Lundquist et al. (48) heated ATCA in strong basic solution to open the ring structure of ATCA and produce a thiol group. Bradham et al. (60) then used hydroxymercuribenzoate, and subsequently diphenylthiocarbazone, to produce a colored product that was analyzed spectrophotometrically. Lundquist et al. (48) derivatized ATCA (after opening the ring) with a coumarin derivative and analyzed by HPLC. After using a cation exchange column (discussed above), Logue et al. (45) prepared ATCA for GC-MS analysis by derivatizing with a silylating agent.

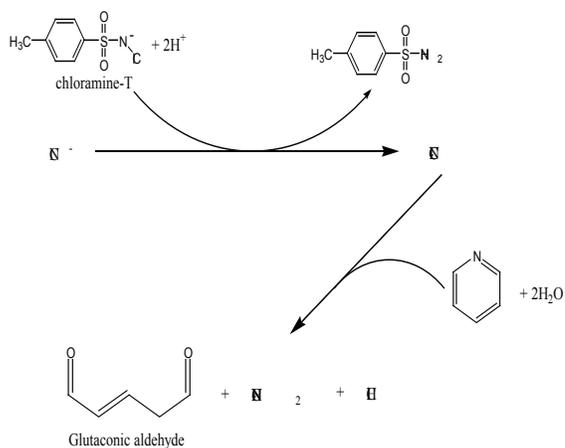
Sample preparation for cyanide-protein adducts involved isolation of the protein of interest with subsequent enzymatic digestion of the adducted protein. Fasco et al. (29) used this technique to analyze protein fragments after digestion with trypsin. Although this method was time-consuming and required highly powerful instrumentation, the authors were able to detect protein-cyanide adducts from the plasma fraction of human blood.

Spectrophotometric, luminescence, and atomic absorption methods

Early spectrophotometric methods of cyanide analysis from biological fluids were often based on the König synthesis (Figure 2) (39, 101-107, 178). König dye synthesis involves oxidation of cyanide using chloramine-T (82, 109, 118, 125), hypochlorite (87, 110, 123) or bromine water (41, 101, 102, 119) to form a cyanogen halide (see Figure 2). The cyanogen halide is then reacted with an aromatic amine (normally pyridine) to produce a glutaconic aldehyde product that is measured in the visible region. These methods have adequate sensitivity, but they lack specificity due to interferences from other chemical species commonly present during the analysis of cyanide, especially thiocyanate and thiosulfate (179). Also, they often require lengthy microdiffusion preparations and the products are unstable. Modifications have been developed that yield more stable reagents and increased precision for this type of reaction (41, 44, 87, 115-117, 119, 125).

Spectrophotometric analysis for thiocyanate is often a variation of the König reaction described above. Hypochlorite and thiocyanate react to form the cyanogen chloride, then either pyridine-malononitrile (76) or barbituric acid-pyridine reagent (95, 118, 123) can be used as coupling agents. Cyanogen chloride can also be reacted with isonicotinic acid to produce a glutaconic aldehyde. Condensation of this aldehyde with two molecules of 1,3-dimethylbarbituric acid produces a dye which can be analyzed spectrophotometrically (89). Other early methods (108) involved oxidation of the thiocyanate to hydrogen cyanide, with aeration into alkali solution, permitting the determination of cyanide as described above for the König reaction. The modified König reaction was also applied to the simultaneous analysis of cyanide and thiocyanate (101,

Figure 2. König reaction for the spectrophotometric analysis of cyanide.



102). Nagashima (109) used the differences in the rates of the reaction of cyanide and thiocyanate with chloramine-T and variations in pH dependence for the simultaneous spectrophotometric determination of cyanide and thiocyanate.

ATCA has also been analyzed spectrophotometrically. Bradham et al. (60) analyzed ATCA from urine as described above. Limitations for this method include interference from a number of species (including cyanide ions) and it is time intensive.

Fluorescence (37, 110-114) methods have also been applied to the determination of cyanide in biological fluids. As with the spectrophotometric methods, fluorescence methods also require extraction techniques to isolate cyanide and eliminate interferences from blood. A number of sensitive fluorometric assays to determine cyanide, free of thiosulfate interference (a problem with a number of spectrophotometric methods) have been developed with greater sensitivity than spectrophotometric methods (110, 111, 114). One specific fluorometric method of analysis of cyanide is reaction of cyanide with NDA and taurine to create a highly fluorescent molecule that can be used for the fluorometric determination of cyanide (37, 159). Cyanide from whole blood has also been determined by flow injection chemiluminescence (180). Acidification and distillation (as mentioned above) was used to separate cyanide from interfering whole blood components and a microchip based reactor was used to mix reagents to produce chemiluminescence.

Although non-chromatographic fluorescence methods for the determination of thiocyanate and ATCA have not been published, the possibility of derivatization with fluorescent derivatizing agents exists. For instance,

3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one and coumarin dyes have been used effectively for the HPLC-fluorometric determination of thiocyanate (163) and ATCA (48), respectively. Cyanide-protein adducts have not been analyzed by fluorescent methods.

Atomic absorption (AA) methods are indirect methods of cyanide or thiocyanate analysis. A metal is added to a sample and a metal complex is formed with the analyte. This complex either precipitates or is extracted into an organic solvent and subsequently analyzed by AA. For example, cyanide has been analyzed by complexing an iron(II)-phenanthroline, and extracting the complex in chloroform for subsequent AA analysis (181). Chattaraj and Das (127) used this technique with flame AA by forming a complex with copper to determine thiocyanate from biological fluids. This technique has also not been used to determine ATCA or cyanide-protein adducts.

Electrochemical, ion-selective electrode, and biosensor methods

Many electrochemical methods for the detection of cyanide and thiocyanate exist, but few of these methods have been applied to their analysis in biological samples (15, 90, 131, 134, 135, 182). Benefits of using electrochemical methods are high sensitivity (for some methods) and quick analysis time. However, they can be subject to multiple interferences from many organic and inorganic ions, including sulfide, ClO_4^- , NO_2^- , N_3^- , and I^- (130, 183). Electrochemical methods can also be hampered by narrow working concentration ranges and may require large sample sizes (132, 137). Westley and Westley (130) used a silver rotating disk electrode, and a dropping mercury electrode for the voltammetric determination of cyanide and thiocyanate in biological samples, including plasma, tissue, and whole blood. Electrochemical detection

can also be used for analysis of cyanide and thiocyanate with ion chromatography (133).

Polymeric membrane-based ion selective electrodes (ISEs) have been developed to address some of the issues limiting electrochemical analysis of cyanide and thiocyanate (21, 132, 136, 183-188). ISEs can exhibit rapid response, high sensitivity, wide linear range, low cost, and they are usually simple to operate. The polymeric membrane in ISEs contains an ion carrier that interacts selectively with the analyte. To selectively analyze cyanide or thiocyanate, the ion carrier must strongly interact with the analyte anion and weakly interact with other anions. This interaction is often enhanced by the use of a metal-ligand interaction. Although many ISEs exist, they are somewhat more limited for anions compared with cations (183).

ISEs have been developed for cyanide analysis, but few have been used for analysis of cyanide from biological samples. This is due to their interaction with multiple ion interferences. If these interferences can be removed, then ISE methods can be used for the determination of cyanide from biological samples. In fact, the standard method for analysis of cyanide from fish tissues is based on ISEs (184). For this method, tissue samples are prepared by homogenizing, acidifying, and distilling internal organs of fish species (similar to those mentioned above in the *Sample preparation and storage* section). Cyanide present in the homogenized tissue is converted to HCN and captured in an alkaline solution following distillation. A cyanide ISE is then used to analyze for cyanide based on its interaction with silver. Another ISE test using gold disc electrodes coated with a sulfonated tetrafluoroethylene copolymer was recently developed by Lindsay and O'Hare for the analysis of cyanide in blood without sample pretreatment (185).

Thiocyanate, due to its lipophilicity, is highly suited for electrostatic interaction techniques, and ISEs have been used successfully for the selective determination of thiocyanate (21). ISEs have been described that exhibit good agreement with values determined by ion exchange chromatography (136) or spectrophotometric methods (132, 183, 186, 187), and were used to analyze thiocyanate from serum (136), urine (132, 183, 186-188), and saliva (132, 183, 186, 188). These include an ISE based on crystal violet thiocyanate or methylene blue thiocyanate in nitrobenzene (136), and a highly selective thiocyanate polymeric membrane sensor that contained a nickel(II)-azamacrocyclic complex coated on a graphite electrode (132). The Ni(II)-electrode showed a great enhancement in selectivity coefficients and detection limits from previously reported electrodes and was successfully used for the analysis of thiocyanate in urine, saliva and milk. Other electrodes which demonstrated good selectivity for thiocyanate in biological samples were a poly(vinyl chloride) (PVC) membrane electrode based on a nickel-hexaazacyclotetradecane derivative (186), a PVC membrane electrode with an unsymmetrical nickel(II) macrocyclic complex as an ion carrier (183), and a graphite electrode based on iron phthalocyanine membranes with sodium tetrphenylborate as a lipophilic anionic additive (187).

Many biosensors (small detection devices normally based on biological activity toward an analyte) exist for the determination of cyanide, including microbial cyanide sensors, sensors based on the enzyme inhibition of cyanide, and sensors based on cyanide degrading enzymes. Most biosensors have advantages of being portable, low cost, easy to use, and can have high selectivity. Limitations of biosensors include degradation of biological components that make up these sensors,

inconsistent electrochemical signals, and difficulty producing sufficient quantities and activities of enzymes or microbes on which these sensors depend. Most of the biosensors developed for cyanide analysis have not been applied to the analysis of biological samples, although a method for the determination of cyanide in fish was recently described (15). Organs of the fish were homogenized with NaOH and a fungal enzyme extract was used to produce formate from metal-cyanide complexes. Then formate dehydrogenase and nicotinamide adenine dinucleotide (NAD⁺) were added to convert the formate into CO₂, which reduced NAD⁺ to NADH. NADH was monitored spectrophotometrically and used to determine the amount of cyanide in the sample.

Currently, biosensors have not been applied to thiocyanate detection from biological samples. Also, electrochemical and biosensor methods have not been applied to analyze for ATCA and cyanide-protein adducts from biological samples.

Liquid Chromatography

The complex nature of biological matrices, small concentrations of cyanide and its metabolites, and the high number of species that interfere with spectrophotometric, luminescent, and electrochemical methods have necessitated analysis of cyanide and its metabolites by more powerful methods. Liquid chromatographic techniques can determine trace amounts of an analyte and can efficiently separate analytes from interfering components in the matrix. Chromatographic techniques, both liquid and gas, also have the ability to simultaneously analyze for cyanide and thiocyanate. For these reasons, they have gained popularity in analysis of cyanide and cyanide metabolites in biological samples.

Three types of liquid chromatography have been used to analyze cyanide: reverse-phase high-performance liquid chromatography (RP-HPLC) (36, 46, 48, 80, 84, 88, 93, 159, 160, 162-164), ion chromatography (IC) (69, 95, 97, 98, 133, 138, 164-169, 189-191), and capillary electrophoresis (139). RP-HPLC methods are common, but generally require pretreatment steps for each anion or multiple post-column reagents. Ion chromatography is often used for thiocyanate analysis, and although these methods are categorized separately, ion chromatography is often a modified RP-HPLC method (i.e., a column modifier is added to the mobile phase to create an ionic stationary phase). Some common detectors used in the liquid chromatographic analysis of cyanide or its metabolites include spectrophotometric (46, 89, 95, 160, 166), fluorescence (37, 84, 88, 93, 159, 163, 164), electrochemical (162, 165, 168, 191), or mass spectrometric (29, 80) detection.

Several groups have adapted the spectrophotometric detection of cyanide and thiocyanate in blood and urine based on the König reaction to RP-HPLC (89, 95, 161, 166). This reaction has also been used for HPLC with fluorometric detection. Toida et al. (84) analyzed cyanide in blood at picomole levels with HPLC and fluorometric detection by using a variant of the König reaction, replacing pyridine with pyridine-barbituric acid. Fluorescence detection was also used for the determination of cyanide in human erythrocytes and whole blood using RP-HPLC with pre-column derivatization with NDA and taurine (88). A number of other fluorometric HPLC methods have been developed for the analysis of cyanide and its metabolites from biological fluids (69, 88, 93, 159, 163). Thiocyanate has also been analyzed using RP-HPLC with fluorometric detection. Tanabe et al. (93) used HPLC with fluorometric detection to determine

thiocyanate in saliva and serum based on the formation of fluorescent cerium (III) from cerium (IV) by a redox reaction. RP-HPLC has also been used for the simultaneous detection of cyanide and thiocyanate. Using pentafluorobenzylbromide as derivatizing agent, Liu and Yun (46) simultaneously determined cyanide and thiocyanate in blood and milk. Mass spectrometric detection was used by Tracqui and Tamura (80) after microdiffusion sample preparation followed by derivatization with NDA and taurine for HPLC-MS analysis of cyanide in blood.

ATCA and cyanide-protein adducts have also been analyzed with RP-HPLC (29, 48). Lundquist (48) used HPLC with fluorometric detection for the determination of ATCA in urine. Although this method was time consuming, it was able to detect ATCA in the urine of smoking individuals. RP-HPLC with mass spectrometric detection has been used to determine cyanide and blood-protein adducts. Fasco et al. (29) used RP-HPLC with tandem mass spectrometric detection to analyze cyanide-adducted proteins from human plasma. The method involved isolation and enzymatic digestion of cyanide-adducted human serum albumin.

A number of authors have used ion exchange (or ion interaction) chromatography to analyze biological fluids for thiocyanate because of the ease of thiocyanate analysis. Chinaka et al. (69) used NDA derivatization of cyanide with an ion exchange column, allowing the simultaneous determination of cyanide and thiocyanate. Lundquist et al. (95) used an ion exchange column with visible spectrophotometric detection for the determination of thiocyanate in serum and urine. Connolly et al. (98) used ion interaction LC with UV detection to analyze thiocyanate in urine. Other authors have coated normal reverse-phase HPLC

columns or used ion-pairing agents to analyze thiocyanate. Examples of this type of analysis include, a RP-HPLC column coated with cetyldimethylamine (97, 169), a zwitterionic micellar-coated stationary phase (189), and bovine serum albumin as the stationary phase with tartaric acid as the eluent (190). Brown et al. (164) used a cetylpyridinium coated reverse-phase column to create an ion-exchange column for the analysis of thiocyanate from rainbow trout plasma in a pharmacokinetic study of thiocyanate exposure. Cookeas and Efstathiou (191) successfully analyzed thiocyanate in saliva with flow injection and ISE detection using a cobalt-phthalocyanine modified carbon paste electrode. ISEs have also been used to detect thiocyanate in urine following ion chromatography (133).

Capillary electrophoresis (CE) has been used successfully for the analysis of thiocyanate in biological fluids (139). Glatz et al. (139) analyzed blood, urine, and saliva samples for thiocyanate with CE and spectrophotometric detection no sample preparation aside from dilution. CE methods for cyanide, ATCA, and cyanide-protein adducts have not been suggested.

Gas Chromatography

One of the most common methods for analysis of cyanide, thiocyanate, and more recently ATCA is gas chromatography. Common detectors used for analysis of cyanide or its metabolites are the electron capture detector (ECD) (66, 70, 81, 140, 141, 151, 154, 156, 192), nitrogen-phosphorus detector (NPD) (33, 62, 63, 78, 79, 86, 124, 144-147, 150, 152, 155), and mass spectrometric (MS) detector (45, 83, 94, 148, 153, 158). Although most of these methods are used for detection of cyanide in blood some groups have applied gas chromatography techniques for other biological matrices and for the detection of thiocyanate and ATCA.

For the detection of cyanide from biological matrices, no specific derivatization is necessary as HCN is volatile. Therefore, the most common pre-analysis step in GC analysis of cyanide is sampling of cyanide from the sample head space. Either equilibrium or dynamic headspace methods can be used to prepare a sample for GC analysis (63, 66, 70, 79, 81, 94, 124, 141, 143, 149, 152, 158). Another pre-analysis step for cyanide analysis is cryogenic oven trapping, which has been used to trap liberated HCN into headspace with high resolution and sensitivity (86, 147). Because thiocyanate and ATCA are non-volatile, they require chemical modification (normally derivatization) to allow analysis by GC. Chemical modification is also necessary for analysis of cyanide by ECD (because of its poor response) or for simultaneous analysis of cyanide and thiocyanate. Therefore, a number of pretreatment steps have been developed to facilitate the analysis of cyanide, thiocyanate, and ATCA by GC.

In GC analysis, the NPD permits the sensitive and specific detection of nitrogen-containing compounds, and has been used for the detection of cyanide and thiocyanate in plasma, urine and saliva (79, 83, 145, 146, 150). However, this detector may be unstable at times, and is less sensitive than some other types of GC detectors. This has led to the creation of analytical methods that take advantage of more stable and sensitive detectors such as the ECD and MS. When using ECD detectors in GC analysis of HCN, derivatization is required before analysis. Derivatization of cyanide has been performed by alkylation of cyanide (83) or the conversion of HCN into cyanogen chloride by choramine-T oxidation (Figure 2) (70, 151, 156). Kage and coworkers (83) used GC-ECD to simultaneously analyze cyanide and thiocyanate using an extractive alkylation technique.

One of the most sensitive methods for analysis of cyanide, thiocyanate, and ATCA is GC-MS (83, 94). With MS detection, stable isotope standards (e.g. ^{13}CN for ^{12}CN) can be used to correct for matrix effects common to cyanide and cyanide metabolites. This can eliminate the need for standard addition techniques and matrix matching. Dumas et al. (94) used stable isotope standards with GC-MS and head space analysis to analyze cyanide concentrations. Kage and co-workers also used GC-MS to analyze both cyanide and thiocyanate simultaneously (83).

ATCA has also been analyzed by GC-MS. Logue et al. (45, 157) analyzed ATCA in plasma and urine using GC-MS by first converting the non-volatile metabolite into a volatile form using trimethylsilyl-trifluoroacetamide. Cyanide-protein adducts have not been analyzed by GC-MS.

Suggested procedures for delayed analysis of biological samples

For cyanide analysis, a sample should be collected quickly after exposure and cyanide analysis should be performed as soon as possible because of the rapid detoxification of cyanide from blood samples (discussed above). However, if analysis of cyanide cannot be performed quickly and storage of biological samples is necessary, the following should be considered:

- 1) *Volatility and nucleophilicity of cyanide.* As described above, HCN is volatile and cyanide ion is nucleophilic. Tightly sealed vials, low temperatures, high pH, and the addition of preserving agents are common procedures that have been used to prevent evaporative loss of cyanide. Storing samples at low temperatures is extremely important to reduce evaporative loss, and slow biochemical reactions. However, there

are many discrepancies in the literature when evaluating the stability of cyanide in biological fluids under various conditions (43, 87, 131, 172, 179). Generally, nucleophilic losses are reduced by adding sequestering agents (e.g., hydroxocobalamin) or chemicals that produce sequestering agents (e.g. sodium nitrite to produce methemoglobin) (87, 123, 193). One method found to improve cyanide stability is the addition of silver ions to biological samples (87).

- 2) *Cyanide concentration varies in biological components.* Cyanide in blood primarily resides in erythrocytes (red blood cells) (64, 82, 87, 174, 194) by binding to methemoglobin, forming cyanomethemoglobin. Cyanide may also be present in plasma, especially if cyanide concentrations exceed erythrocyte concentrations (82, 87). To ensure accurate cyanide concentrations when analyzing blood, collection containers that contain anticoagulants (e.g., heparin) should be used to prevent clotting. Analysis of cyanide from tissues requires knowledge of the behavior of cyanide in specific organs since the enzyme that catalyzes conversion of cyanide to thiocyanate has highly variable concentrations depending on the organ. Therefore, in specific organs, there will be little to no cyanide because of extremely fast metabolism to thiocyanate.
- 3) *Potential for cyanide formation during storage.* Artfactual formation of cyanide may also occur in biological samples depending on storage conditions (77, 172-174). It has been suggested that oxyhemoglobin (175), thiocyanate oxidase (173, 174), and white blood cells (77) may oxidize thiocyanate to cyanide and these reactions are dependent

on the temperature and pH of the sample. Microorganisms may also be responsible for cyanide production and low temperature storage will help to eliminate their growth (173).

These considerations are common to all the analytical methods for analysis of cyanide from biological samples and certainly contribute to discrepancies in similar studies in the literature. For post-mortem analysis of cyanide, production and transformation of cyanide must be considered when interpreting results along with other considerations discussed above (74, 195-198).

Some of the same issues must also be considered for thiocyanate, as a number of problems with storage of samples to be analyzed for thiocyanate have been found (56). This may be due to interconversion of thiocyanate and cyanide over time and the removal and production of thiocyanate by biological processes other than cyanide detoxification (24, 58). It has been suggested that ATCA is not involved in other biological processes and it has been found to be stable during storage (18, 45, 47, 48, 59). While ATCA may not have the storage issues of cyanide and thiocyanate, there is little information on its toxicokinetics, which currently limits its use as a biomarker for cyanide exposure. Also, cyanide-protein adducts have recently been discovered, and therefore little information about the toxicokinetics and stability of these adducts is known.

Conclusions

The analytical determination of cyanide and its metabolites is not an easy task due to chemical properties, biological activities, and limited research. Numerous methods have been developed and each has its own advantages and disadvantages. However, they

have all provided insight into the verification of cyanide exposure from analysis of biological samples. Table 3 provides a comparison of analytical techniques for analysis of cyanide or its metabolites based on sensitivity, specificity, sample size, capacity, expertise necessary to perform the method, and cost. Some other key pieces of information should also be considered prior to choosing a method to perform: 1) Was preservation of cyanide and its metabolites during storage addressed?; 2) Were typical interferences for the biological matrix of interest removed?; 3) Were analysis procedures that could result in the loss of cyanide or its metabolites used (i.e., heating or acidification)?

For the analysis of cyanide, the largest inconsistency in the literature is analysis with different preservation techniques. Regardless of the analytical method, a preserving technique needs to be considered so that accurate concentrations of cyanide can be found (75, 87, 112, 123, 128, 197-200). If cyanide is analyzed, biological samples should be collected and analyzed as soon as possible for confirmation or refuting cyanide exposure. One also needs to consider that all biological samples will contain endogenous levels of cyanide (and its metabolites). Therefore, baseline levels of the analyte measured should

be known prior to concluding an exposure occurred. Problems with direct analysis of cyanide, including short half-lives, artifactual formation of cyanide, and interconversion of cyanide and thiocyanate, contribute to difficulties in the analysis of cyanide for all analytical methods designed to determine cyanide in biological samples.

While cyanide is most often analyzed to determine cyanide exposure, one should consider using analytical techniques that analyze for cyanide metabolites as well as those that analyze for cyanide directly. Although cyanide metabolites may offer longer half-lives, they also have a number of drawbacks. For thiocyanate, the main drawback is large and variable background concentrations in biological samples. Other disadvantages include are interconversion of cyanide and thiocyanate, and the use of thiocyanate by other biological processes not directly related to cyanide metabolism. For ATCA and cyanide-protein adducts, the main drawback is the limited amount of research available on toxicokinetics and relationships of these metabolites to cyanide exposure. Care should be taken when choosing an analytical method to consider not just the parameters of the analytical method but also the toxicokinetics of cyanide and its metabolites.

Table 3. Analytical Methods to determine cyanide and its metabolites in biological fluids.^a

Technique ^b	Sub-category	Analyte ^c	Matrixes	Sensitivity ^d	Specificity ^d	Sample Size ^d	Capacity ^d	Expertise ^d	Cost ^d	Refs.
UV-Vis	None	CN, SCN, ATCA	Blood, Urine, Saliva	Low	Low	0.5-1 mL	Medium	Low	Low	(41, 44, 60, 61, 76, 77, 82, 87, 89, 101-105, 108, 109, 115-119, 123, 178, 179)
	Fluorescence	CN	Blood	Medium	Medium	0.5-1 mL	Medium	Medium	Low-Medium	(77, 110-114, 124)
Electro-chemistry	Chemiluminescence	CN	Blood	Medium-High	Medium	3 µL	Medium	Medium	Low	(180)
	None	CN, SCN	Blood, Saliva, Tissue	Medium-High	Medium-High	1-5 mL	Medium-High	Low	Very Low-Low	(15, 21, 90, 130-137, 182, 183, 185-187, 201, 202)
AA	Indirect AA	SCN	Blood, Saliva	Medium-High	Medium	1 mL	Low	Medium	Medium-High	(127)
Biosensor	None	CN, SCN	Blood, Urine, Saliva	Medium-High	Medium-High	1-5 mL	Medium-High	Low	Very Low-Low	(15)
	RP-HPLC UV	CN, SCN	Blood, Urine	Medium	High	10-100 µL	High	Medium-High	Medium	(89, 95, 160, 166)
IC	RP-HPLC fluorescence	CN, SCN, ATCA	Blood, Urine, Saliva	High	High	10-100 µL	High	High	Medium	(37, 84, 88, 93, 159, 163, 164)
	RP-HPLC electrochemical	SCN	Urine	Medium-High	High	10-100 µL	High	Medium-High	Medium	(162, 165, 168)
	RP-HPLC mass spectrometric	CN	Blood	Very High	Extremely High	10-100 µL	High	Very High	High	(80)
	RP-HPLC MS-MS	CN-protein adduct	Blood	Extremely High	Extremely High	10-100 µL	Low	Extremely High	Extremely High	(29)
	IC	CN, SCN	Blood, Urine, Saliva	Medium	High	1-100 µL	High	High	Medium	(97, 167, 169)
GC	GE	SCN	Blood	High	High	1-10 nL	High	High	Medium-High	(138, 167)
	GC-NPD	CN, SCN	Blood, Urine, Saliva	High	Very High	1-10 µL	Medium	High	High	(33, 62, 63, 78, 79, 86, 124, 144-147, 150, 152, 155)
	GC-ECD	CN, SCN	Blood, Urine, Saliva	Very High	Very High	1-10 µL	High	High	High	(66, 70, 81, 140, 141, 151, 154, 156, 192)
	GC-MS	CN, SCN, ATCA	Blood, Urine, Saliva, Tissue	Very High	Extremely High	1-10 µL	High	High	Very High	(45, 83, 94, 148, 153, 158)

^a This table is meant to give a general overview of analytical techniques to analyze cyanide and its metabolites along with a general idea about parameters specific to each analysis technique. Parameters of specific methods within a particular analysis technique may be outside of those listed.

^b UV-Vis – ultraviolet visible spectrophotometry, AA – atomic absorption, IC – liquid chromatography, RP – reverse phase, MS-MS – tandem mass spectrometric detection, IC – ion chromatography, CE – capillary electrophoresis, GC – gas chromatography, NPD – nitrogen phosphorus detector, ECD – electron capture detector.

^c CN – cyanide, SCN – thiocyanate, ATCA – 2-amino-2-thiazoline-4-carboxylic acid.

^d These parameters are related to the general instrumental technique used and not each individual method of analysis.

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Background Paper on Cyanide Detection Tests for Live Fish

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Purpose of this Document

The purpose of this document is to provide an overview of cyanide detection testing as it applies to the capture of live reef fish. We have provided an introduction to the live fish aquarium and food trades, as well as the techniques that utilize cyanide solutions to capture fish. We have provided a summary of cyanide detection systems currently used to determine whether commercial fish were caught with cyanide. Finally, this document includes a summary of cyanide detection methods used in medical and environmental testing. As part of this last element, we have identified scientists and other technical experts that are most knowledgeable and experienced in cyanide detection methods.

We hope this document provides sufficient background information for those parties interested in applying existing methods of cyanide detection to the development of a detection system that can be used in marine fish. To help achieve this goal, we have presented a summary of the criteria necessary for development and implementation of an effective cyanide detection test.

Introduction

The sale of live fish from the ocean is a valuable international trade. The trade includes aquarium fish that are sold through the hobby market, as well as fish sold live to restaurants for human consumption (also called the live reef food fish trade, or LRFFT). According to an American Marinelife Dealers Association survey, in 1995, about 10 million individual marine specimens were sold in pet

stores throughout the United States for a total value of \$103.2 million (Marine Aquarium Council). The United States imports nearly half of the marine aquarium organisms sold throughout the world. Other major importers are Germany, France, Netherlands, United Kingdom, and Japan. Hong Kong is the largest consumer of live reef food fish and accounts for 60% of the world trade (Mak, Yanase et al., 2005 citing Johannes and Riepen 1995).

Live marine organisms are harvested from the coral reefs of Southeast Asia, the Pacific Islands, South Asia, Indian Ocean, Australia, Hawaii, Mexico, Florida, the Caribbean, Brazil, East Africa, and the Red Sea. The two dominant countries that supply the ornamental fish trade are Indonesia and the Philippines, accounting for more than half of the global marine ornamental fish trade (Marine Aquarium Council).

In an effort to ensure that this industry has a long, healthy future, many governments, non-profit organizations, and industry members are committed to measures that protect the environment that produces the fish. One organization engaged in such initiatives is the Marine Aquarium Council (MAC), whose mission is to “conserve coral reefs and other marine ecosystems by creating standards and certification for those engaged in the collection and care of ornamental marine life from reef to aquarium.” A variety of other organizations focus on issues related to the capture of live fish sold to restaurants.

Cyanide Use in Fish Capture

As in any business, there is a great deal of competition and as a result tremendous pressure to develop efficient techniques of fish capture. One such capture technique involves the use of the toxic substance cyanide. Some collectors use cyanide to temporarily stun fish and make them easier to catch.

Cyanide (CN) occurs naturally in the environment and some food substances. Industrial activities such as metal processing, electroplaters, gold-mining facilities, oil refineries, power plants, and solid waste combustion can also release cyanide into the environment (Eisler, 1991).

Hydrogen cyanide is toxic to the majority of living matter and fish are no exception (Eisler, 1991 citing Marrs and Ballantyne, 1987). Cyanide is a general respiratory poison, although uptake can also occur through ingestion or dermal absorption (Eisler, 1991 citing Towil et al., 1978). In high doses, death can occur within minutes (Eisler, 1991). In the live fish trade, the amount of cyanide used is relatively low and recovery is quick.

Where Is Cyanide Fishing Used?

According to Jones (Jones, Kildea et al., 1999) cyanide fishing has been confirmed in at least 15 countries or island territories including Indonesia, Malaysia, Maldives Islands, Papua New Guinea, the Philippines, Sri Lanka, Thailand, and Vietnam (Johannes and Riepen, 1995 cited in Barber and Pratt, 1997; McManus, Reyes et al., 1997). The use of cyanide is illegal in most countries; however, enforcement resources are low and cyanide continues to be a regular practice. The use of cyanide is also illegal in Australia, yet the law appears to be sufficiently enforced, making the Australian live reef food fishery cyanide-free (Barber and Pratt, 1997)

Fish Collection Techniques Using Cyanide

While fishing for aquarium and live food fish, collectors place sodium or potassium cyanide tablets in small plastic bottles filled with seawater (Jones and Steven, 1997). Detergent, milk, or kerosene can also be added to the squirt bottle to increase the visibility of the solution and to reduce the amount of cyanide needed. This has the benefit of reducing costs as well as lowering fish death from cyanide overdose (Erdman and Pet, 1999).

The collectors squirt the cyanide solution in the direction of a fish, which may be hiding on or inside the coral reef. Additionally, anecdotal reports state that in some cases fish collectors must break large areas of coral to retrieve the stunned fish hiding in the coral (Mouse, Pet-Soede et al., 2000; Fahrudin, 2003). Divers then collect the asphyxiated fish and place it in a bag of fresh seawater for transport. Throughout the journey, collectors will exchange the seawater frequently (Johannes and Riepen, 1995). Other collection techniques include placing cyanide inside portions of bait or as part of a fish paste used to attract fish (Jones and Steven, 1997).

How Much Cyanide Is Used?

The Marine Aquarium Council website reports that the International Marinelifelife Alliance (IMA) tested 48,000 fish in the Philippines to find that 25% of aquarium fish destined for the United States and Europe, and 44% of live groupers and humphead wrasse going to Hong Kong, were caught using cyanide.

The concentration of cyanide in the squirt bottles is not known. Analyses of cyanide squirt bottles seized on cyanide fishing vessels in Indonesia indicated concentrations of 2 g/l (Pet and Djohani, 1998). An analysis by Fahrudin (Fahrudin, 2003), reported that fishermen from two islands in Indonesia used between 50-60 grams of cyanide per

day. One cyanide tablet (2 g) is mixed with approximately 3 liters of water in a plastic bag or bottle. Based on this information, Fahrudin (Fahrudin, 2003) calculated that the resulting cyanide concentration is approximately 6.67 g/l.

Other studies suggest that cyanide concentrations in squirt bottles vary by almost an order of magnitude (Jones, Kildea et al., 1999): 13 g/l (Pet, 1997), 100 g/l (Barber and Pratt, 1997), 30 ± 120 g/l (Johannes and Riepen, 1995).

What Species Do Fishers Catch with Cyanide?

In the marine fish aquarium trade, species targeted by cyanide fishing include nearly all coral fish species. However, the collectors first target the high price fish species, such as emperor angelfish (*Pomacanthus imperator*), blue surgeonfish (*Paracanthurus sp.*), and blue ring angelfish (*Pomacanthus annularis*) (Fahrudin, 2003).

In the live food fish trade, common reef fish caught are groupers (Serranidae), wrasses (Labridae), and snappers (Lutjanidae). The humphead (Napoleon) wrasse (*Cheilinus undulatus*), high-finned grouper (*Cromileptes altivelis*), and giant grouper (*Epinephelus lanceolatus*) are traded only in small volumes but are particularly valued (Johannes and Riepen, 1995; Mak, Yanase et al., 2005)

Impact of Cyanide Fishing

It is hard to find documentation on or to estimate the impact of cyanide collection on individual fish or on the environment surrounding the fish. In addition, the aquarium fish trade is highly dynamic and collection techniques change quickly, depending on cost of materials, ease of use, and individual ability (Erdman and Pet, 1999). Below is a summary of research results that estimate the impact

of cyanide fishing on the fish collected as well as the surrounding environment.

What Does Cyanide Do to the Fish?

As a general summary, hydrogen cyanide is toxic to the majority of living matter (Eisler, 1991 citing Marrs and Ballantyne, 1987). Specifically, cyanide appears to be acutely toxic to aerobic organisms at concentrations greater than 0.1 - 0.3 mg/l causing death within 96 hours (Doudoroff, 1980 cited in Mak, Yanase et al., 2005).

Eisler (Eisler, 1991) conducted an extensive literature review to evaluate the effect of cyanide on a large list of organisms including: freshwater and marine aquatic organisms, birds, and mammals. The results indicated that fish were the most sensitive aquatic organisms tested under controlled conditions. Fish that were exposed to a range of 5.0 - 7.2 $\mu\text{g/l}$ free cyanide per liter displayed significant adverse non-lethal effects, including reduced swimming performance and inhibited reproduction (Eisler, 1991). Mak et al. (Mak, Yanase et al., 2005) report that a 10 μM solution of cyanide is non-lethal to fish and equivalent to what fish collectors use on the reef.

There is also anecdotal evidence from fish collectors and the marine aquarium industry that fish caught without cyanide live longer and have lower mortality rates throughout shipment. Along with increased mortality comes the need to catch more fish to respond to the demand for aquarium fish. Ultimately, the increased death caused by cyanide fishing increases the potential of overfishing.

Metabolism of Cyanide in Fish

Hydrogen cyanide enters a fish's bloodstream through the gills and intestine and is rapidly distributed to other body tissues. Cyanide is toxic to fish because it interferes with oxygen

metabolism by blocking the key enzyme system, cytochrome oxidase (Metzler, 2001), and blocks enzymatic pathways in the liver (Solomonson, 1981). Some of the effects, such as blocking enzyme functions, are irreversible and lead to the death of the fish (Way, Leung et al., 1988). Once inside the fish tissue, cyanide reacts with thiosulfate in the presence of rhodanese to produce the comparatively nontoxic thiocyanate. The thiocyanate is excreted in the urine. Rapid detoxification enables animals, such as fish, to ingest high, sub-lethal doses of cyanide (Eisler, 1991).

Chu (Chu, Liu et al., 2001) used attenuated total reflectance and transform infrared microspectroscopy to evaluate the molecular mechanisms of cyanide toxicity. The results showed changes in structural conformation of biomolecular/protein components of gill tissue and ultimate loss of function.

It is not possible to definitively identify the half-life of cyanide in marine fish. However, because of the volatile nature of cyanide, it is known that cyanide breaks down very rapidly (Logue, Kirschten et al., 2005). This is particularly true in fish and even more so in a salt-water environment. Logue et al. (Logue, Kirschten et al., 2005) state that depending on the route and duration of exposure, in humans cyanide is typically eliminated from blood within 20 minutes post-exposure.

Exposure of marine fish to a 10 μM concentration of potassium cyanide for 15 minutes is considered to be a sub-lethal dose (Mak, Yanase et al., 2005). Preliminary tests to examine cyanide concentrations present in marine fish after exposure to cyanide identified a range of 0.04 ppm to 1.02 ppm (Hodgson, pers. com.).

Eisler (Eisler, 1991) reported baseline levels of cyanide in freshwater fish at less than 1 $\mu\text{g}/\text{kg}$ or 1 ppb fresh weight in gills. However, values up to 50 $\mu\text{g}/\text{kg}$ occurred occasionally in fish found in natural streams (Eisler, 1991). Cyanide concentrations in fish from streams that were deliberately poisoned with cyanide ranged between 10 and 100 $\mu\text{g}/\text{kg}$ (10-100 ppb) total cyanide whole body fresh weight (Wiley, 1984). Total cyanide concentrations in gill tissues of salmonids under widely varying conditions of temperature, nominal water concentrations, and duration of exposure ranged from about 30 $\mu\text{g}/\text{kg}$ fresh weight to greater than 7,000 $\mu\text{g}/\text{kg}$ (Holden and Marsden, 1964; Eisler, 1991).

What Does Cyanide Do to the Coral Reef?

It is even more difficult to assess the impact of cyanide use on the reef environment. Ultimately, the dose of cyanide experienced by corals and surrounding organisms is a function of cyanide concentration, the proximity to target fish to the cyanide source, and the local hydrological conditions. Under conditions of cyanide fishing, corals are likely to experience initially high (10^{-1} to 10^{-2} M), rapidly fluctuating concentrations of cyanide that ultimately fall to very low levels (10^{-5} to 10^{-6} M) (Jones and Steven, 1997). In some cases, water currents can dissipate cyanide and carry the toxin away from the reef. However, research using dyed water showed that water can be trapped in stagnant zones behind coral heads (Wolanski and Jones, 1980). Under such conditions, cyanide fishing could result in coral mortality (Jones and Steven, 1997).

Early research showed that photosynthesis and calcification of staghorn corals were inhibited at concentrations greater than 1×10^{-5} M cyanide (Chalker and Taylor, 1975). More recently, Jones and Steven (Jones and Steven, 1997) showed that respiratory rates of *Pocillopora damicornis* were inhibited by

10–90% following exposure to cyanide but recovered to pre-exposure levels within 1–2 hours after transfer to clean seawater. These researchers also observed that corals died after exposure of cyanide at the highest doses used by fish collectors. After medium doses, the corals lost their symbiotic algae resulting in discoloration or bleaching. After exposure to the lowest doses they lost zooxanthellae but not in sufficient numbers to cause noticeable discoloration.

Cervino (Cervino, Hayes et al., 2003) also found that cyanide exposure can be lethal to corals. Cervino (Cervino, Hayes et al., 2003) exposed a variety of coral and anemone species in the lab and in the field to the following concentrations of cyanide solution for 1–2 minutes: 50, 100, 300, and 600 mg/l. Upon exposure, corals and anemones immediately retracted their tentacles and mesenterial filaments, and discharged mucus containing zooxanthellae. Gel electrophoresis techniques found changes in protein expression in both zooxanthellae and host tissue. Both corals and anemones showed an immediate increase in mitotic cell division of their zooxanthellae, and a decrease in zooxanthellae density. In contrast, zooxanthellae cell division and density remained constant in controls. Other changes included gastrodermal disruption, mesogleal degradation, and increased mucus in coral tissues. Zooxanthellae showed pigment loss, swelling, and deformation.

Cervino (Cervino, Hayes et al., 2003) reported mortality occurred at all exposure levels, and concluded that exposure to cyanide causes mortality to corals and anemones, even when applied at lower levels than those used by fish collectors. Cervino (Cervino, Hayes et al., 2003) also stated that even brief exposure to cyanide caused slow-acting and long-term damage to corals and their zooxanthellae.

Jones et al. (Jones, Kildea et al., 1999) measured the effects of cyanide on coral photosynthesis through a series of experiments conducted in the field. These measurements were made in situ and in real time using a submersible pulse amplitude modulation (PAM) fluorometer. In *Stylophora pistillata*, exposure to cyanide resulted in an almost complete cessation in photosynthetic electron transport rate (Jones, Kildea et al., 1999).

Jones et al. (Jones, Kildea et al., 1999) concluded that, based on these studies, cyanide-induced bleaching has been documented in five species of corals. The species studied represent a variety of coral types, including encrusting, massive, and branching growth forms. This experimental evidence of bleaching is supported by observations (Erdman and Pet-Soede, 1996) who report bleached and dead corals surrounding holes or recesses on reefs where cyanide fishing had occurred (Jones, Kildea et al., 1999).

Cyanide Fishing in Context

This document outlines that exposure to cyanide presents a temporary stress on fish, corals, and surrounding organisms. Overall, the literature suggests that these impacts have cumulative, potentially long-lasting effects; however they are difficult to quantify. Conservation and Community Investment Forum (CCIF) presented one estimate of overall reef-degrading capacity of the cyanide fishery for food fish. In Indonesia the loss of live coral cover is approximately 0.052 m² per 100 m² of reef per year (Conservation and Community Investment Forum, 2001).

It is important to include the work of some researchers that state that the aggregate impact of cyanide fishing is minimal. Mouse et al. (Mouse, Pet-Soede et al., 2000) state that the toxicity of cyanide to corals under experimental conditions is, in itself, no proof

of degradation on the scale of a reef. For example, Mouse et al. (Mouse, Pet-Soede et al., 2000) state that blast fishing accounts for a loss in live coral cover of 3.75 m² per 100 m² of reef each year (Pet and Pet-Soede, 1999), which is about 75 times more than one estimate of cyanide impact, and still about 5–6 times more than the ‘worst-case’ estimate for the loss of coral cover due to cyanide fishing for food fish.

Other Toxins Used to Catch Fish

Cyanide is the most widely used chemical in marine fish capture. However, other poisons are utilized as well. Clove oil (or eugenol in its purified form) is a moderately well-known anesthetic for small fishes and crustaceans (eg. Soto and Burhanuddin, 1995; Munday and Wilson, 1997; Erdman and Pet, 1999). Quinaldine is another narcotic associated with the ornamental industry. It is reported to be less dangerous than cyanide, but capable of killing fish during collection when concentrations are high or exposure time is long (Randall, 1987). Bleach, formalin, and gasoline were reported to be used occasionally to catch aquarium fish in Puerto Rico in the early 1990s (Sadovy, 1992), but the extent to which they are used elsewhere is not known. There are no data available that quantify the extent of use of these chemicals.

Non-Toxic Fish Collection

An alternative to fishing with toxic substances such as cyanide is to catch fish with small nets or to use hook and line. Numerous regional organizations train collectors in how to use these non-destructive methods. Despite the training, collectors have been slow to switch to using nets because they can earn more using cyanide. In some cases it is possible to pay the collector a premium price for fish caught in a non-destructive manner (Barber and Pratt, 1997).

Existing Cyanide Detection Tests

Because of the fact that cyanide can damage the marine environment and potentially decrease the mortality of fish sold in the commercial trade, the use of cyanide to capture fish is illegal in nearly all countries where it is commonly used. In order to enforce the law and monitor the presence of cyanide in fish, the International Marinelife Alliance-Philippines (IMA) and the Philippine Bureau of Fisheries and Aquatic Resources (BFAR) have developed a cyanide detection test (CDT) to determine the presence of cyanide in live-caught fish. The American Analytical Testing Laboratory supported the development of the test (Mak, Yanase et al., 2005).

The first CDT laboratory was established at BFAR headquarters in Manila in 1992 and now includes six laboratories and three monitoring inspection stations. The IMA CDT uses ion selective electrodes (ISEs) to detect the concentration of cyanide in the distillate (Mak, Yanase et al., 2005, see Table 1). In brief, internal organs of a fish, where cyanide is rapidly absorbed, are homogenized with water in a blender. Then the homogenate is strongly acidified in a 1-hour reflux distillation. If cyanide is present in the sample, it is liberated as hydrogen cyanide (HCN) and absorbed into a sodium hydroxide solution. Finally, the presence of cyanide in absorbing solution is detected using ISE. Each CDT lab is capable of running at least 20 tests per day. Each test takes a total of 1.5 hours including the procedures described as above. After the test, the result is given back to the owner of the source of the sample in the form of certification (Barber and Pratt, 1997; Mak, Yanase et al., 2005).

Possibilities to Improve Cyanide Detection Tests

Recently, researchers from the University of Hong Kong simulated and reviewed the

existing cyanide detection methods used in fish samples. The aim was to answer the following questions: (1) is the current cyanide detection method appropriate and effective and (2) what kinds of improvements can be done to achieve a higher sensitivity and accuracy? (Mak, Yanase et al., 2005).

The results indicate that the current method could not measure cyanide concentration below 1×10^{-5} M. However, much lower cyanide concentration in seawater was enough to suffocate a 500 g marine food fish (Mak, Yanase et al., 2005). Therefore, the researchers concluded that the cyanide detection method currently used was not sensitive enough for the determination of cyanide traces in post cyanide-exposed fish. In other words, these results indicate the amount of cyanide necessary to kill fish is well below the amounts that can reliably be determined using the existing procedures (Mak, Yanase et al., 2005).

Ultimately, an effective cyanide detection test will include the following criteria:

- Sufficient sensitivity to detect low quantities of cyanide in marine fish hours and perhaps up to days after exposure
- Ability to distinguish between background levels of cyanide exposure versus concentrations resulting from illegal fishing practices
- Use of technology that is available in countries where marine fish collection occurs
- Ability to detect cyanide exposure prior to metabolic processing by fish
- Technology that is possible for use by non-specialized field staff
- Ability to replicate the procedure in many locations

- Ability for rapid reporting of results to industry and government representatives
- Affordable costs
- The ability to test fish at the location of import country — which could take place several weeks after original exposure to cyanide

It may be necessary to develop a hybrid approach that utilizes a less sensitive screening method in the field. Field-based test samples that indicate a positive result for cyanide use can be followed up with more sensitive and verifiable lab-based tests done on the same fish sample. The techniques that are appropriate in the field include colorimetric and some bioassay procedures. If the test includes chromatography-related methods such as gas chromatography and high performance liquid chromatography/mass spectrometry, it will be necessary to conduct this work in a laboratory.

Preliminary Work to Improve Cyanide Detection in Fish

Mak and Law (Mak, Law et al., 2005) conducted preliminary experiments to develop a biosensor system and satisfy the necessary detection criteria. Their methods include the pretreatment of cyanide with cyanide hydrolase and then detection by spectrophotometric formate sensor. The researchers report that the system has a detection limit at $7.3 \mu\text{mol/l}$ although theoretically it is possible to increase the sensitivity of the biosensor by injecting a larger volume of the pre-incubated sample (i.e. more formate) into the measuring cell.

The researchers identify the following advantages of this system over other potential cyanide detection tests:

- cyanide hydratase converts the toxic cyanide into non-toxic products for measurement
- the combination of enzymes makes the system highly specific
- there is no need for vigorous sample pre-treatment
- no use of hazardous chemicals

To take this work a step further, Mak et al. (Mak, Yanase et al., 2005) used the microbial sensor system to measure cyanide levels in marine fish. They exposed fish to sub-lethal doses of 10 μ M potassium cyanide in seawater for 15 minutes and then transferred the fish to clean seawater for recovery. After different recovery times (0-180 minutes), fish were tested for residual cyanide using the biosensor system. Results are expected in a forthcoming publication (Mak et al., in prep).

Cyanide Testing Protocols Used in Non-Fish Settings

Because of the fact that cyanide is a toxic poison and the fact that it can be an environmental pollutant, there is a large scientific literature on cyanide detection.

Table 1 summarizes the most common cyanide detection tests as well as advantages and disadvantages of each test. Table 2 presents a sampling of cyanide detection tests in human, fish, and mouse blood, urine, and tissue samples. In addition, Table 2 presents some publications reporting on a variety of cyanide detection tests.

It is interesting to note that there is a large amount of activity in the U.S. federal government to develop rapid cyanide detection in humans in order to prepare for a potential bioterrorist attack using cyanide. It is possible to explore these methods and experts to find avenues through which to

improve the current cyanide detection system for marine fish.

The current method used to test cyanide concentrations in fish employs the Ion Selective Electrode (ISE) methodology. This method is not highly sensitive and it is subject to a tremendous amount of interference with samples found in seawater. Tables 1 and 3 present a wide range of methodologies, each with varying degrees of cost, sensitivity, and potential effectiveness for marine fish samples.

Because cyanide breaks down so quickly in fish, it may be necessary to develop a test that measures a by-product of cyanide. Some of the possibilities to explore in this arena include:

- Thiocyanate. Thiocyanate is a non-toxic by-product of cyanide degradation.
- Other biological by-products such as:
 - o formate (Mak, Law et al., 2005)
 - o a cyanide/hemoglobin adduct (Kobleski, pers. com.)
 - o 2-aminothiazoline-4-carboxylic acid (ATCA) (Logue, Kirschten et al., 2005)
 - o a gill protein that is changed upon cyanide exposure (Chu, Liu et al., 2001)
- Cytochrome oxidase — dose-related reductions in cytochrome c oxidase activity were detected in various organs of rats exposed to oral doses of potassium cyanide (Ikegaya, Iwase et al., 2001). This marker was suggested as a method of diagnosis for samples taken within two days post-mortem.

- Plasma lactate — elevated plasma lactate concentrations, resulting from the shift to anaerobic metabolism, have been used to assess the severity of cyanide poisoning in humans (Baud, Borron et al., 2002)

Thiocyanate is the major metabolite of cyanide, and a number of sensitive methods are available for its measurement in biological fluids (see Table 2 and Logue, Kirschten et al., 2005). Thiocyanate can be a good marker for cyanide exposure because it is non-volatile and can be analyzed in blood, urine, and saliva. However, analytical recovery of thiocyanate from whole blood is not quantitative and thiocyanate concentrations in blood varied inconsistently during storage at various temperatures over a period of two weeks (Logue, Kirschten et al., 2005). In addition, thiocyanate may be formed by other modes of metabolism besides cyanide intoxication or metabolism (Logue, Kirschten et al., 2005).

These same limitations to measuring thiocyanate as a marker of cyanide concentration could equally be true for other metabolites such as formate and ATCA. All systems that are developed to detect cyanide will have to demonstrate that they can differentiate between background, endogenous sources of cyanide, and that their concentrations directly correlate with external cyanide exposure.

One lab (Logue, Kirschten et al., 2005) is developing a promising possibility for cyanide detection by looking at the cyanide metabolite 2-aminothiazoline-4-carboxylic acid (ATCA). ATCA is stable for months in biological samples at freezing and ambient temperatures (Logue, Kirschten et al., 2005). The method analyzes synthetic urine and swine plasma through cyanide derivatization and subsequent gas chromatography–mass

spectrometry (GC–MS) analysis. The study identified a detection limit of 25 ng/ml. This lab has also conducted preliminary tests of this method of fish samples (Logue, pers. com.). However, further research is needed to verify the background levels of ATCA in fish samples, the metabolic clearance rate of ATCA in fish, and the correlation between cyanide exposure and subsequent ATCA concentrations.

Summary

The publication of the Mak et al. (Mak, Yanase et al., 2005) paper has initiated a discussion regarding the possibility of developing an improved cyanide detection test for marine fish. However, it is important to acknowledge that the detection of cyanide, cyanide compounds, or cyanide metabolites in fish tissues is quite difficult. Once exposed to cyanide, the fish rapidly incorporate and convert cyanide into thiocyanate (SCN⁻). Shortly thereafter, the thiocyanate is excreted. The half-life of the cyanide is extremely short. As a result, detection in the fish tissues will be a factor of the amount of time that passes before a test can be performed. Mak et al. (Mak, Yanase et al., 2005) point out that it may be that detection of total cyanide in fish would occur only under “optimal conditions” such as extreme cyanide exposure followed by rapid implementation of a cyanide detection test.

Table 1. Summary of Cyanide Detection Test Methods

Method	Advantages	
<p>Microbial Biosensors Use of microbial cells or enzymes to convert cyanide into by-products. The electrochemical signal produced by the cyanide by-products correlates with original cyanide concentration.</p>	<ul style="list-style-type: none"> - inexpensive - simple to operate - products are non-toxic - fast processing time - no interference 	<ul style="list-style-type: none"> - samples easily affected by nutrients - limited life time of electrode - sample preparation may be required (2 hrs.)
<p>Ion Selective Electrodes Ion Selective Electrodes (ISE) are membrane electrodes that respond selectively to ions in the presence of others. The voltage is theoretically dependent on the logarithm of the ionic activity, according to the Nernst equation.</p>	<ul style="list-style-type: none"> - does not require extensive sample preparation - inexpensive - equipment is durable in field settings 	<ul style="list-style-type: none"> - electrode loses sensitivity with use - requires frequent recalibration - results vary with temperature - iodide levels in seawater will cause interference - prone to interference from other ions
<p>Fluorometric Fluorescence spectroscopy or fluorometry is a type of spectroscopy used for analyzing compounds that have the ability to fluoresce. Generally, this fluorescence is directly proportional to the concentration of the material in question.</p>	<ul style="list-style-type: none"> - reduced interference - high sensitivity 	<ul style="list-style-type: none"> - sample preparation by microdiffusion required - impurities may diminish fluorescence - interference potential still exists without a pre-separation mechanism .e.g. chromatography
<p>Colorimetric Colorimetry is a method to measure the concentration of a known constituent of a solution by comparison with colors of standard solutions of that constituent.</p>	<ul style="list-style-type: none"> - rapid detection - cost effective 	<ul style="list-style-type: none"> - interference from thiosulfate ions - sample preparation often required - interference
<p>Mass Spectrometry Mass spectrometry measures the masses and relative concentrations of atoms and molecules by using the basic magnetic force on a moving charged particle.</p>	<ul style="list-style-type: none"> - very sensitive and selective - provides confirmatory structural information 	<ul style="list-style-type: none"> - machines may not be available in source countries - relatively expensive - needs expertise to run and maintain instrument
<p>Electron Capture Detection ECD detects electron-absorbing components in the output stream of a gas chromatograph. The concentration is proportional to electron capture.</p>	<ul style="list-style-type: none"> - selective detection combined with chromatographic detection 	<ul style="list-style-type: none"> - see comments on GC
<p>Nitrogen/ Phosphorous Detection Detects electron-absorbing components in the output stream of a gas chromatograph.</p>	<ul style="list-style-type: none"> - selective detection combined with chromatographic separation 	<ul style="list-style-type: none"> - See comments on GC

Method	Advantages	Disadvantages
<p>High Performance Liquid Chromatography/Mass Spectrometry (HPLC-MS) The combination of a mass spectrometer and a liquid chromatograph makes a powerful tool for the detection of trace quantities of non-volatile compounds.</p>	<ul style="list-style-type: none"> - “gold standard” analytical technique for quantitative analysis of complex biological and environmental samples - high sensitivity - may be most suitable to investigating metabolites of cyanide exposure - potential of detecting evidence of earlier cyanide exposure 	<ul style="list-style-type: none"> - may require derivatization (sample pre-treatment) of analyte of interest (i.e. cyanide metabolites)
<p>Gas Chromatography (GC)</p>	<ul style="list-style-type: none"> - very sensitive and selective 	<ul style="list-style-type: none"> - possibly expensive - machines may not be available in source countries - needs expertise to run and maintain instrument
<p>Direct GC Gas chromatography is a type of chromatography in which the mobile phase is a carrier gas, usually an inert gas, and the stationary phase is a microscopic layer of liquid on an inert solid support, inside a column.</p>	<ul style="list-style-type: none"> - minimal sample preparation but, depending on analyte of interest, extraction of sample may be required 	<ul style="list-style-type: none"> - see above comments on GC
<p>Headspace GC Headspace analysis is most suited for the analysis of the very light volatiles in samples that can be efficiently portioned into the headspace gas volume from the liquid or solid matrix sample. Complex sample matrices, which would otherwise require sample extraction or preparation, or be difficult to analyze directly, are ideal candidates for headspace since they can be placed directly in a vial with little or no preparation.</p>	<ul style="list-style-type: none"> - minimal/no sample preparation - relatively easy to use once validated protocol is established 	<ul style="list-style-type: none"> - see above comments on GC

Table 2. Summary Published Cyanide Detection Tests

Paper	Method	Matrix	Detection Limits
(Mak, Yanase et al., 2005)	Microbial Biosensor	Fish Tissue	$1 \times 10^{-5} - 1 \times 10^{-4}$; = 1.1 μM = 0.286 ppm
(Mak, Yanase et al., 2005)	Microbial Biosensor	Fish Tissue	7.3 $\mu\text{mol/l}$
(Mak, Law et al., 2005)	Microbial Biosensor	Fish Tissue	7.3 $\mu\text{mol/l}$
(Ikebukuro, Shimomoura et al., 1996)	Microbial Biosensor	Aqueous Solutions	$0-1.5 \times 10^{-5}$
(Groom and Luong, 1991)	Microbial Biosensor	Aqueous Solutions	$5 \times 10^{-6} - 1 \times 10^{-3}$
(Lee and Karube, 1995)	Microbial Biosensor	Aqueous Solutions	0.1^{-1} ppm in 50 mM phosphate buffer (pH 8)
(Smit and Cass, 1990)	Biosensor (Peroxidase-based)	Aqueous Solutions	1.0×10^{-7} (limit of detection)
BFAR Test for Live Fish Trade (Romero, Rodolfo et al., 2003)	Ion Selective Electrodes (ISE)	Fish Tissue	Greater than 1×10^{-5} M (Mak et al., 2005)
(Egekeze and Oehme, 1979)*	Ion Selective Electrodes (ISE)	Blood and Liver	5 g/l
(Murphy, Schantz et al., 2006)	Gas Chromatography (Headspace)/ Mass Spectrometry	Blood	60 ppb with uncertainty of 8%; = 0.7 ng/g = 0.007 $\mu\text{g/g}$
(Ishii, Seno et al., 1998)	Gas Chromatography (Direct)	Blood	2.0 ng/ml
(Levin et al, 1990)*	Gas Chromatography (Headspace)	Not Available	Not Available
(Odoul et al., 1994)*	Gas Chromatography (Headspace)	Not Available	Not Available
(Tsuge, Kataoka et al., 2000)	Gas Chromatography Spectrophotometry	Blood and Saliva	0.05 μM
(Hughes, Lehner et al., 2003)	Gas Chromatography (Headspace) and Spectrophotometry	Equine Blood	2 ng/ml
(La Forge et al, 1994)*	Mass Spectrometry	Blood	0.07 g/ml

Paper	Method	Matrix	Detection Limits
(Tomoda and Hashimoto, 1991)*	Mass Spectrometry	Blood	0.4 g/ml
(Cruz-Landeira et al., 2000)*	Mass Spectrometry	Urine	28 ng/ml
(Morgan and Way, 1980)*	Spectrophotometry	Blood	0.1 ppm
(Tracqui, Raul et al., 2002)	High Performance Liquid Chromatography - Mass Spectrometry	Whole Human Blood	5.0 ng/ml (abstract only)
(Sano, Takezawa et al., 1989)	High Performance Liquid Chromatography – Fluorometric	Urine	30 pmol/ml
(Deschamps, 2004)	Colorimetric	Aqueous Solutions	0.2 ppm and higher, not effective for detection of 0.1 ppm or lower
(Rella, Marcus et al., 2004)	Colorimetric	Aqueous Solutions	Greater than 1.0 ug/ml
(Fligner, Luthi et al., 1992)	Colorimetric	Human Whole Blood	0.2 mg/l
(Ganjeloo, Isom et al., 1980)	Colorimetric, Fluorometric	Mouse Blood	0.025 ppm (0.001 umol/ml)
(Pettigrew and Fell, 1973)	Colorimetric (with microdiffusion)	Blood	
(Sano, Takezawa et al., 1989)	Spectrofluorimetric	Blood and Urine	0.03 nmol/ml
(Sano, Takezawa et al., 1989)	Fluorometric	Whole Human Blood and Urine	0.03 nmol/ml (abstract only)
(Chinaka, Takayama et al., 1998)	Ion Chromatography/Fluorometry	Cyanide and Thiocyanate in Blood	

The following are some methods to detect thiocyanate, a biological by-product of cyanide			
(Liu and Yun, 1993)	High Performance Liquid Chromatography -Mass Spectrometry	Thiocyanate in Saliva	2 ng
(Pettigrew and Fell, 1973)	Colorimetric	Thiocyanate in Blood and Urine	Approximately 0.07 ppm ¹
(Chanttaraj and Das, 1992)*	Spectrometry (flame absorption)	Thiocyanate in Blood, Urine, Saliva	4 ng/ml
(Li et al, 1993)*	Spectrophotometry	Thiocyanate in Blood	0.3 ug/ml
(Chen et al., 1994)*	Gas Chromatography (Headspace)	Thiocyanate in Urine, Saliva	0.0115 nmo (in 0.2 ml)
(Michigami et al., 1992)*	Ion Chromatography	Thiocyanate in Urine, Saliva	20 ng/ml
(Tominaga and Midio, 1991)*	Spectrophotometry	Thiocyanate in Urine	2.5 umol/l

* Data obtained from published literature and not original paper

Table 3. Key Cyanide Detection Facts

Half-life of cyanide in fish	Unknown, but possibly 1-3 days
Concentration of cyanide in squirt bottles before exposure	Wide range: 6.6-120 g/l (see text)
Concentration of cyanide in fish after exposure	0.04 ppm to 1.02 ppm (Hodgson, pers. com.)
Detection limit of Ion Selective Electrodes currently used	Greater than 1×10^{-5} M (Mak, Yanase et al., 2005)
Background level of cyanide in fish (freshwater)	less than 1 µg/kg or less than 1 ppb (Eisler, 1991)
Background level of cyanide in humans	50 ppb
Toxic level of cyanide in humans	2.5 ppm (Kobeleski, pers. com.) 0.1 – 0.3 mg/l (Doudoroff, 1980 cited in Mak, Yanase et al., 2005).
Cyanide concentration that produces non-lethal, negative impacts in fish	5.0 - 7.2 µg/l (Eisler, 1991)

Table 4 lists experts identified to date related to cyanide detection. We predict there are two possible ways that some of these experts can support efforts to develop a cyanide detection system in marine fish:

- Conduct research to develop the laboratory protocols necessary for cyanide detection
- Serve as peer reviewers and advisors to evaluate and advise the protocol development

The following individuals expressed interest in reviewing a request for proposals and potentially conducting analytical research to develop the protocols:

- Dr. Reinhard Renneberg Laboratory, Hong Kong University of Science and Technology
- Dr. Brian Logue Laboratory, South Dakota State University
- A.C.S Laboratory, Cleveland, Dr. Mike Makelov
- Haereticus Laboratory, Clifford, VA, Dr. Craig Downs

The following individuals have expressed interest in providing advisory services in the form of review of a request for proposal or review of analytical techniques. Several of these individuals have a personal connection with the issue and are, or have been, aquarium hobbyists.

- Dr. Stephen Lowes, Advion and owner of coral growing business
- Dr. Bob Kobeleski, Center for Disease Control and lead to develop cyanide response network
- Dr. Steve Baskin, Aberdeen Proving Ground
- Dr. Terry Mudder, Editor, *Cyanide Monograph*

The far left column of the table provides an informal indication of the current status of communication with the expert. “C” indicates an intention to call the individual; “F” means we are attempting to find the experts; “Em” means we have sent an email; “X” means we have completed contact; “M” means we have left a message; “H” means we have had communication and the contact is on hold for the moment; and “P” means this expert is a priority for future activities.

Table 4. Cyanide Experts

	Name	Affiliation	Contact Information	Notes
P	Dr. Stephen Lowes	Advion	607-266-0665, ext. 106/306 607-844-5587 (home) lowess@advion.com	Rec. by Jack Henion. Owns coral growing business and is chemist for Advion. Happy to help on this project.
P	Bob Kobeleski	CDC Research Chemist	770-488-4686 rmk9@cdc.gov	Leading team of public health labs to develop emergency cyanide terrorism response network. Very knowledgeable.
	Karen Mak	Hong Kong University Renneberg Laboratory	Karen@ust.hk	Developed biosensor system to detect cyanide in fish.
P	Steve Baskin	Aberdeen Proving Ground	410-436-2378 steven.baskin@amedd. army.mil	Medical/chemical aspects of chemical warfare. Rec. by Karen Murphy. Very knowledgeable. Writing a review of cyanide detection methods.
P	Brian Logue	South Dakota State University, Department of Chemistry and Biochemistry	954-629-2134 brian.logue@sdstate.edu	Developed test to detect ATCA- a CN metabolite. Uses GC-MS method and trying to develop an HPLC method. Set of 10 samples takes 4 hrs (15 minutes for each GC-MS sample).
G	Terry Mudder	Editor, <i>Cyanide Monograph</i> .	Cyunara@aol.com www. Cyantists.com	Mining and cyanide expert. Very helpful, as is the website. Also Lead of Cyantists, Coordinator of Cyanide in the Environment Conference.
C	Tim Van Weingarden Ask for other experts.	ACZ labs	ACZ Laboratories, Inc. Phone: 970-879-6590 Toll Free: 800-334-5493 Fax: 970-879-2216	Terry Mudder says best lab in country that works on cyanide. Ask for lab manager. Lab says it does not carry out R&D, so can't develop a protocol.
G	Ben Blount	Lead Research Chemist VOC and Perchlorate Laboratory, CDC	770-488-7894 bkb3@cdc.gov	Bob Kobeleski says he is looking at thiocyanate in urine. Has a proposal with a pharma house to look at CN exposure in fire victims.
G	Mike Makelov	A.C. S. Labs Cleveland, Ohio	216-642-8515 headspace@earthlink.net	Physical chemist. Absolute expert on headspace GC.
X	Barry Logan	State Toxicologist, Washington State	206-262-6000 barry.logan@wsp.wa.gov	Worked to developed a quick test of human blood with Cyantesmo paper strip.
C	Steve Burden	Retired from BP ran CN testing lab, was a marine biologist	419-657-6304 419-657-6853	Maybe would collaborate with Makelov.

X	Jerry Thomas	CDC	770-488-7279 ciq1@cdc.gov	Board-certified toxicologist. Working on a fire exposure antidote. Interfaces with Merck Germany. Rec. by Bob K.
		Research Triangle Institute	Charlie Sparacino Spar@rti.org 919-541-6581 Em 8/22/06 Edgar Cook, retired (M 8/21/06 540-885-2945 919-541-6000	Working with EPA
Em	Steve Borron	UT?	Sborron@intoxicon.com Boron@uthscsa.edu	Setting up surveillance of CN in hour fire victims. Rec. by Jerry Thomas.
X	Jack Henion	Adivon	607-266-9162 x308 henionj@advion.com	Veterinary toxicologist. Former professor at Cornell Vet school. Has his own company. Might know someone in field.
X	Todd Anderson	Texas Tech Institute of Environment and Human Health	806-885-0231 todd.anderson@tiehh.ttu.edu	Ecotoxicologist with big perchlorate lab in fish tissue. Like CN, it is an ion that you measure with same techniques. He uses ion chromatography method.
C	Alex Krynitsky	FDA Research Chemist	301-436-2098	Measures perchlorate in food.
Em	Yasuo Seto	National Research Institute of Police Science, 4 th Chemistry Section	Seto@nrrips.go.jp 81-471-35-8001	
F	James Way	Previously at Texas A&M	Jlway@medicine.tamu.edu	Was on HHS panel (ask him about others).
F	Bryan Ballantyne	Retired. Previously, Dow or Union Carbide, Director of Applied Toxicology	Charleston, West Virginia	True expert on Cyanide. Wrote "Toxicology of Cyanide." Determining if still active.
C	Tim Miller Morgan	Oregon State University, Asst. Professor, Aquatic Pets	Hatfield Marine Science Center 541-867-0265 541-270-4218 tim.miller-morgan@oregonstate.edu	Collaborating with MAC. They have done some cyanide testing in cattle.
F		University of Michigan		According to Paul Holthus they have a specialty of cyanide testing in animals.

F	Gary Isom	Purdue University		Recommended by Steve Baskin.
F	Joe Borowitz	Purdue University		Recommended by Steve Baskin.
F	Gary Rockwood	Aberdeen Proving Ground		Recommended by Steve Baskin.
F	Randal Baselt	Biomedical Publications Corporation. Forensic toxicologist.	650-344-2256	
X	Prof. Paul Bowser	Cornell College of Veterinary Medicine, fish pathologist	607-253-4029 prb4@cornell.edu	Rec. by Jack Henion. He is happy to help, it is a bit off-field for him.
Em	Michael Peat	Editor, <i>Journal of Forensic Sciences, toxicologist</i>	Michael.peat@att.net Jfs.editor@att.net	Previous Director of Center for Human Toxicology
Em	Bruce Goldberger	Editor, <i>Journal of Analytical Toxicology</i>	Rocky Point Labs Office: 352-265-0680 Bruce-goldberger@ufl.edu	
H	Ed Pfannkock	Gerstel Laboratories	Support@gerstelus.com 410-204-7251	Providing technical support to cyanide terrorism network. Assisted in equipment development. Makes the automated sampler (cost is \$30K) used by CDC.
F	Ronald Eisler	National Fish and Wildlife Service, perhaps now at NOAA	Reisler@fisheries.org 301-897-8616	Evaluated toxic environmental impact of NFWS cyanide application program.
X	JR Deschamps	U.S. Navy, Laboratory for Structure and Matter	Deschamps@nrl.navy.mil	Field test for water.
Em	Craig Downs	Haereticus Laboratory	haereticus1@hughes.net Phone/Fax: 434-263-5740	Has conducted cyanide test on fish in the Middle East.
X	Bill McShane	Toxicologist, CDC	770-488-4311 wmm9@cdc.gov	Participant on CDC cyanide terrorism project. Not able to talk to us.
H		Cyanide Reagent Strip		Used by EPA Not good for field
H	ES Deocadiz	Environmental Management Bureau (EMB), DENR, Philippines		Wrote ASEAN marine water quality criteria for cyanide.
H	Peter Scott	Previously with MAC		Managed process with MAC and Merck to evaluate current BFAR test.

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COUNTRY REPORTS

Destructive Fishing Practices in Vietnam: Current Status with a Focus on Cyanide Fishing

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Introduction

Vietnam has a coastline of over 3260 km with an Exclusive Economic Zone of over 1 million square kilometers. The fisheries sector plays a vital role in the economic and social development of Vietnam. Of a population of 84 million the livelihood of 8 million people depends on fisheries resources as the household primary income source and an additional 12 million get part of their income or subsistence from fisheries (MOFI and WB, 2005). Although over the past decade, the fisheries sector has achieved considerable growth this has not been without concerns of sustainable development. Such problems include overfishing in the coastal area, degradation of the marine environment and coastal resources, including the use of destructive fishing methods, and the lack of effective resource management. The use of destructive fishing methods yields short-term economic benefits for fishers but endangers the long-term sustainability of fishing and other reef-dependent industries such as ecotourism.

A report released in 2002 documented that destructive fishing practices, including the use of poison and dynamite, threaten as much as 85 percent of Vietnam's reefs (WRI, 2002). The leading threats to Vietnam's reefs in 2004 continued to be over-fishing and destructive fishing. Based on 2003-2004 data, most reefs surveyed in Vietnam have less than 25% live

coral cover (Tun et al., 2004).

Vietnam ranks among the top five countries most impacted by climate change (Dasgupta et al., 2007). Eliminating destructive fishing in Vietnam, including cyanide fishing, will assist to enhance resistance and resilience to climate change of Vietnam's coastal resources by alleviating the overall pressures on the system, giving it more flexibility to mobilize its natural defenses.

This report details the current status of management and use of destructive fishing practises in Vietnam with a particular focus on cyanide fishing. To alleviate ecological impacts of destructive fishing practises particularly cyanide use, a series of urgent measures is suggested covering the legislative framework, improving law enforcement and awareness raising.

Overview of destructive fishing practises

In Vietnam four common methods of destructive fishing, which have been illegal in Vietnam since 1989, are:

1. Destructive fishing gear (e.g. illegal mesh size)
2. Explosive fishing (also referred to as blast or dynamite fishing)
3. Electro fishing
4. Poison fishing (mainly cyanide)

The first three of these destructive fishing methods are discussed below. A detailed assessment of cyanide fishing (a frequently used poison for fishing), one of the most commonly used destructive fishing methods used in Vietnam, is provided in section 3.

Destructive fishing gear

The majority of fishing gear used by fishers in Vietnam do not conform to mesh size regulations (Nguyen Chu Hoi, 2008). For example the sizes of trawl cod end, purse seine's bunt net and trammel net are so small that a large proportion of juveniles are caught. A number of harmful fishing gear such as estuary set nets and scoop nets are still used (RIMF (2006). There is scant information on the extent and impact of destructive fishing gear on fisheries resources as well as impacts on bycatch and habitats.

Dynamite/blast fishing

Dynamite or "blast" fishing is one of the most immediate and destructive threats to coral reefs worldwide. Dynamite fishing mainly occurs in the marine environment in Vietnam. As with other methods of destructive fishing in Vietnam there is little quantitative information available on the extent of use of dynamite fishing. A survey in Nha Trang Bay showed that around 10% of manta-tows showed evidence of blast fishing in the form of craters and or other obvious physical damage to reef areas (Vo Si Tuan et al., 2002, cited in Tuan V.S. et al 2005). Based on the fieldwork of the study team and available data a Ministry of Fisheries (MOFI) and World Bank report in 2005 concluded that dynamite fishing is still widespread in some areas in Vietnam, but in areas where the resource has already been severely depleted it has ended (MOFI and WB, 2005). Dynamite has also been recorded as being used in conjunction with push nets where the push net is used to herd the fish and dynamite is

thrown into the herded school of fish (Smith et al., 2003).

Electro fishing

Electro fishing occurs in freshwater and salt water areas in Vietnam. Several kinds of electro fishing are used in Vietnam a number of which are detailed below.

Xiec Dien – electro fishing equipment made with a large Y-shaped wooden scoop net at the end of which are two electric poles that transmit the voltage into the water stunning small shrimp and fish.

Gia nui – electric fishing equipment is attached to a dragnet to enhance its efficiency

Gia tiep – fishers use electric poles between two boats and close to the sea floor affecting a large area.

Incentives to engage in illegal electro fishing have been documented as a low initial investment cost and compared to traditional fishing methods it is less labour intensive and no specific knowledge is needed. This is in contrast to traditional fishing methods where nets are more costly and require specific fishing knowledge of where and when to fish (Strehlow, 2006). The level of enforcement and the fine levels are also not considered a sufficient a deterrent. 2005 MOFI and World Bank report concluded that the use of electric fishing in Vietnam is increasing, destructive and difficult to prevent (MOFI and WB, 2005).

Cyanide fishing

Poisons (Chat doc) are used widely in Vietnam as a method of fishing. One of the most commonly used poisons is Cyanide. Marine aquarium species that are difficult to catch by mesh and scoop nets because they hide in holes and burrows such as Angel fish (Serranidae) and eels (*Anguilla*) are caught by cyanide or anaesthetics (Truong Si Ky, 2006).

The detrimental effects of cyanide fishing on coral reef fish as well as coral are well documented (Rubec et al., 2001, Cervino et al., 2003).

Current status of cyanide fishing

Cyanide fishing in Vietnam is largely driven by two key fisheries:

1. The international trade in live reef food fish
2. The marine aquarium fishery

A report on the status of Vietnam's reefs in 2005 noted that although dynamite fishing had decreased, cyanide fishing had become more popular due to the increased demand of the live fish trade (Tuan et al., 2005). Vietnam is located near to the two biggest live reef fish markets Hong Kong and China - a nation importing up to 90% of food fish worldwide (Tuan et al., 2005).

The distribution of the use of cyanide fishing in Vietnam has not been surveyed in a systematic way. Areas of use of cyanide have only been identified in scattered reports. The use of cyanide is common in reef sites in the north (Co To, Bach Long Vy) and central provincial islands (Ly Son, Cu Lao Cham) due to the availability of cheap cyanide pills (8000 VND each - USD 0.5). Exploitation of coral reef fish using cyanide in Co To Island (Quang Ninh province), has been identified as the major cause of a critical decline in marine aquarium fishery resources (Nguyen Van Quan et al., 2007). Overfishing and use of destructive methods (cyanide, blast and fine mesh nets) for a range of coral reef fisheries has resulted in widespread destruction of reefs in the following provinces in Vietnam: Quang Ninh, Nghe An, Quang Binh, Thua Thien-Hue, Quang Nam, Da Nang, Quang Ngai and Khanh Hoa (See Figure 1) (Chou, 2000).

Hon Mun MPA island communities (Khanh Hoa Province) are home to many poor fisher families whose subsistence includes a decreasing catch supplying the local and international aquarium fish trade. They are responsible for a large proportion of the unsustainable fishing activity near Hon Mun including the use of cyanide and dynamite (GEF Project documentation http://www.gefweb.org/COUNCIL/GEF_C14/vietnam/viethon.pdf). According to local sources cyanide fishing was only introduced into Khanh Hoa between 1994 and 1996. Local government officials believe that Hong Kong-based middlemen and traders supply cyanide to fishermen to guarantee the supply of certain volumes and species (McCullough and Hai, 2001).

In Bach Long Vy Island, the level of cyanide in the sea was on average 0.65mg/l, more than 13 times the permitted limit; 300mg/kg in sediment, 3 times more than Canada's standard; 40mg/kg in seaweed, 20 times more than America's standard; 550mg/kg in abalone, more than 2.5 times America's standard. The coral "jungle" in north-east Bach Long Vy has been significantly destroyed due to the use of cyanide to fish for grouper. The environment surrounding Bach Long Vy Island has been polluted by poisons for a long time, one of the reasons leading to exhausted abalone resources (Nguyen Huy Yet et al., 2004).

This scattered reporting of the use of cyanide use highlights the need for developing a structured project to determine the areas and extent of use in order to inform suitable management responses.

In Vietnam, prices paid to fishers for live coral groupers are three times higher than for frozen (McCullough and Hai, 2001), making the use of illegal cyanide a viable option particularly when fines are relatively low and

enforcement is minimal. There is anecdotal information that middlemen that buy live reef food fish also supply fishers with the cyanide (Strehlow, 2006).

In 2002 it was reported that 80% of the live marine aquarium fish exported from Vietnam die during transport to consuming nations from the detrimental effects of cyanide, ammonia and stress (Rubec and Palacol, 2002). The current percentage of mortalities in live marine aquarium fish from Vietnam is unknown. High mortality rates, due to cyanide capture and poor collection, transport, and handling procedures is a major problem impacting the viability of the marine aquarium and live reef food fish export trade from Vietnam.

In Vietnam little attention is paid to the health of fishers using diving equipment, despite warnings in the media by scientists regarding the harmful effect of using cyanide for fishing and using directly compressed air without filtering while diving (Barber and Pratt, 1997, 1998). Divers in Vietnam have reported feeling drunk for a short time after using too much cyanide (Strehlow, 2006). Little is known about the long term health impacts of cyanide on fishers.

Although there seems to be a good understanding of the negative impacts of cyanide fishing, the economic returns surpass those of traditional fishing, and with little enforcement, this illegal activity persists.

Researchers have worked with local communities in Indonesia and the Philippines to develop cyanide-free net capturing techniques for the marine aquarium trade (Rubec et al., (2001). In order to reduce the impact of cyanide fishing on coral reefs, as well as on diver health, alternatives for

cyanide fishing need to be developed for local Vietnamese fishing communities.

An assessment of the legislative landscape for cyanide management

The Vietnamese government has made various commitments internationally and promulgated legislation domestically to eliminate destructive fishing practises.

International Commitments

In the international arena Vietnam has made numerous commitments to phasing out destructive fishing practices as detailed in Appendix 1. Key commitments include:

- FAO Code of Conduct for Responsible Fisheries (1995) which contains a commitment to end destructive fishing practices
- APEC Fisheries Working Group (1997) - Endorsed a resolution urging “all members of the international community to cease the practise of cyanide fishing”
- WSSD (2002) - Contains a commitment to phasing out destructive fishing practices in the marine environment by the year 2012

Current domestic legislation and policies

A range of domestic legislation has been promulgated relating to destructive fishing in Vietnam (see Appendix 2). A decree strictly forbidding the use of dynamite, poison (cyanide) and electric fishing was first made in 1989 (Chapter II, Article 8 (1) Ordinance on the protection and development of aquatic resources 1989). Despite enactment of this legislation the use of destructive fishing practises still persisted 9 years later, particularly in provinces in central Vietnam. In response to this situation the Prime Minister in 1998 produced a directive “strictly banning the use of explosives, electric impulses and toxics to exploit aquatic resources”, also known as Directive 01 (Directive 01/1998/CT-TTg 2

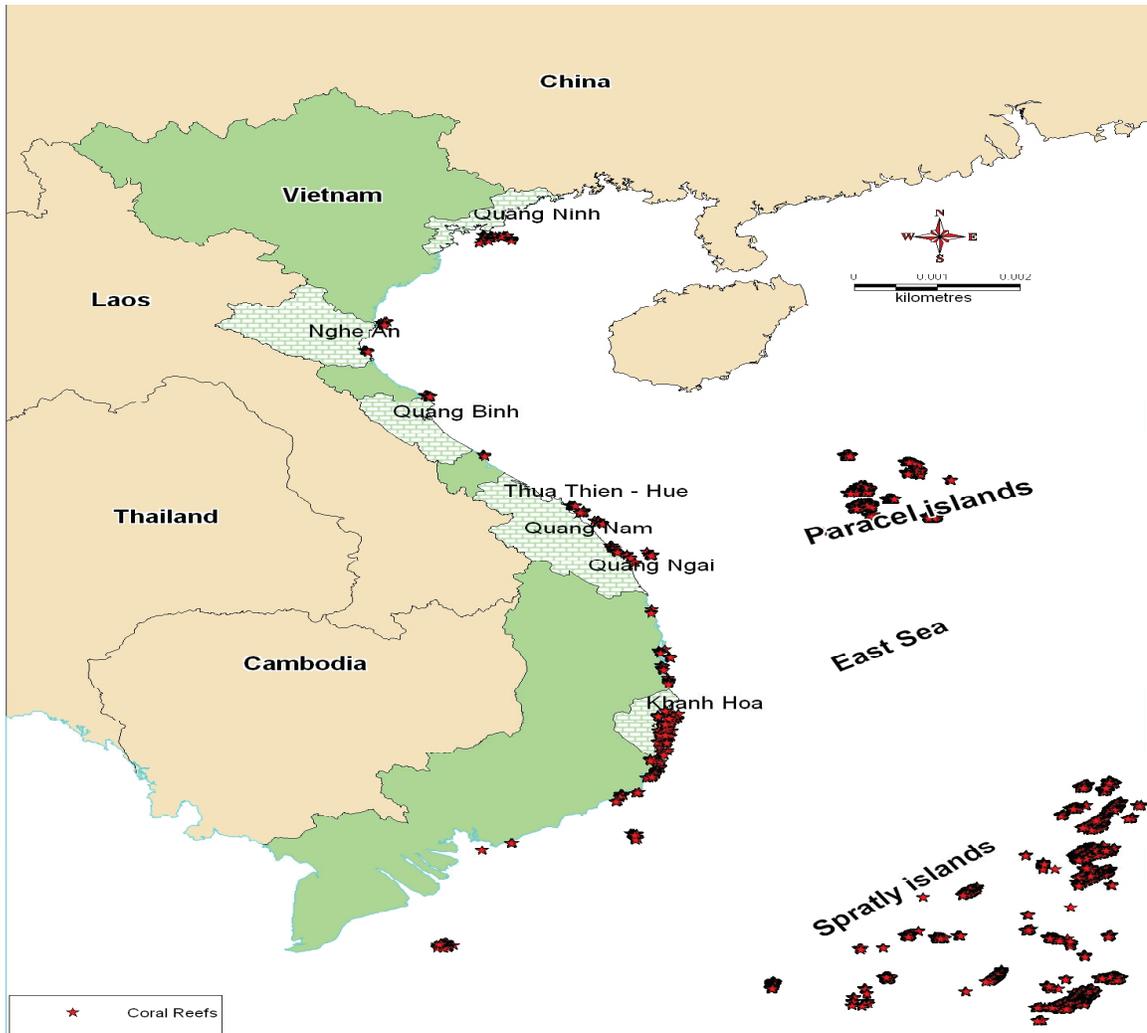


Figure 1: Key provinces with wide spread destruction of reefs due to destructive fishing practices (Source: Chou, 2000)

January 1998). The directive prescribed action on compliance, enforcement and awareness raising. Additionally it directed District Peoples Committees to make plans to survey and classify poor fishers and take measures to assist them in developing aquaculture or alternative sustainable fishing methods (Article 6 Directive 01/1998/CT-TTg).

The use of dynamite, electro and poison fishing declined during the period 1999-2003 following the implementation of Directive

01. However, since 2006, due to declining coastal fisheries resources and higher petrol/oil prices, the use of dynamite, electro and poison fishing has increased. In response to this increase the government of Vietnam, since early 2007, has set up a permanent Steering Committee (also called the Steering Committee 01) to prevent dynamite, electric and poison fishing. The Steering Committee has identified the following important tasks that they have accomplished:

1. Management/guidance to the Ministry of Fisheries (MOFI) (consulted by Steering Committee 01) concerning continued strengthening and implementation of the Directive 01 (promulgated the document 1679/BTS-BVNLTS 26/7/2007). MOFI specifically requests Provincial Peoples Committees to:

- Implement widely and frequently Directive 01 to district and commune levels and people unions.
- Strengthen the Steering Committee from provincial to district and commune levels. The Steering Committees should include some institutions: fisheries, public security/police, military, culture and information. Commune people's committee chairmen should have the main responsibility for implementation.
- Strengthen communications of Directive 01.
- Undertake regular inspections,
- Investigate and propose alternative livelihoods for communities living near coastal, water bodies, lagoons.
- Report to the Steering Committee every 3, 6 and 12 months.

2. Communication, education and training

3. Inspection of implementation of Directive 01 in some local areas

4. Cooperation with DOFI inspectors: in inspecting in the rivers and the sea

The 01 Steering Committee detailed the following solutions for implementing Directive 01 in 2008 including:

- Development of new regulations for the 01 Steering Committee activities
- Strengthening local Steering committees and establishing Steering committees in areas where they have not yet been established
- Review and summary by localities of 10

years of implementation of Directive 01 (1998-2008) to prepare for a National conference expected to be held in the third quarter of 2008 (MARD, 2007)

In order to develop Directive 01 effectively, the Steering Committee 01 was strengthened with the membership of four Ministries: MOFI (recently integrated to MARD), Ministry of National Defence, Ministry of Public Security and Ministry of Information and Communications. This combination of ministries highlighted the important role of information to strengthen community awareness and understanding of banned fishery activities. However this role has not yet been adequately considered at the local level where the illegal fishers are active because of the ineffective collaboration between local stakeholders and communication system.

Although cyanide fishing is illegal, this chemical is still circulated and traded quite openly on the market in Vietnam. Cyanide is not listed in the government decision on chemicals banned from export and import (Decision 05/2006/QD-BCN Article 1 lists chemicals banned from import and export), as cyanide is used widely in industrial production and only referred to as a deleterious waste (Decision 23 QD-BTNMT Article 2 Declare the list of deleterious waste matters). The lack of government regulations on limiting and defining authorized institutions/individuals in the use and trade of poisons has led to the situation where the management of cyanide is difficult to control.

Fish harvested with cyanide are more likely to have a higher mortality rate (Baquero, 1999) and as a result experienced aquarium owners are willing to pay more for higher quality fish (Tsang, 2001). A number of countries have developed facilities that can test for cyanide in fish caught for the marine

aquarium trade. The Bureau of Fisheries and Aquatic Resources (BFAR) in the Philippines have established a Cyanide Detection Test (CDT) to analyze cyanide content in fish and in water. The laboratory issues certifications on analysis result to support regulatory and enforcement laws as well as export requirements (http://www.bfar.da.gov.ph/infocorner/cyanide_detectiontest.htm (accessed 6/03/07); Rubec et al., 2003). There have been a number of calls for the development of a CDT in Vietnam both for the Live Reef Food Fish fishery and the marine aquarium fishery to assist in monitoring the use of cyanide and enforce national laws and regulations (Nguyen Duc Cu, 2001). To date such a system has not been developed.

The USA Lacey Act prohibits the import of any wildlife that has been traded in violation of foreign law. This Act was adopted to assist in controlling the destructive wildlife trade in settings where the environmental laws of originating states could not be enforced in those nations. Thus it is illegal to import into the USA marine aquarium species taken in Vietnam with cyanide. The USA Coral Reef Task Force are investigating cyanide detection tests that can both be used for local management and enforcement authorities in source countries and also a test that could reliably be used several weeks after exposure in order to allow the USA to apply the Lacey Act to suspected illegal imports (CRTE, 2006).

Compliance and enforcement

Despite the existence of clear legislation regarding destructive fishing, the use of destructive fishing gear and methods has not been effectively prevented in Vietnam (Nguyen Long RIMF, <ftp://ftp.fao.org/docrep/fao/007/ad939e/ad939e00.pdf>). The challenges in Vietnam for enforcement of the destructive fishing legislation are:

- The vast geographic area with a coastline of more than 3260km
- A lack of management coordination between National, Provincial and District levels
- A lack of capacity and human resources

Government agencies with the power to enforcement destructive fishing legislation are (Articles 25, 26 & 27 of Decree 128/2005/ND-CP):

- Fishery Inspectors (Administrative and Enforcement)
- The Chairs of Provincial/District People's Committees
- Other Functional Departments (Force of Border Military, Force of People Police, Marine Police)

The administrative structure for management of fisheries enforcement is provided in Figure 2. At a national level, NADAREP is the state authority responsible for implementing law/regulations relating to protecting fishery resources. At the provincial level, the Department of Aquatic Resources Exploitation and Protection (DAREP) is the representative office of NADAREP but is under the Department of Fisheries (DOFI) which has a consultation role to the Provincial Peoples Committee within the fishery domain and concerned policies instead of authority of enforcement. Although NADAREP directly manage the local fishery enforcement inspectorate in terms of line management, key “additives” such as human resources and finance integral to effective enforcement are decided by the local People's Committee.

Due to the above limitation, local fishery enforcement has to work in cooperation with other functional departments (Force of Border Military, Force of People Police, Marine Police), however, the “integrated management” issue is a significant challenge

in Vietnam now and requires more effort and resources to resolve.

At the district/local level, sub-offices of DAREP placed in local areas can be active in the field. However, these offices have limited human resources and enforcement capacity.

At the provincial level, fishery inspectors are the key law enforcers, however most of them are educated in terms of technical domain but lack an understanding of law enforcement. Additionally human resources are limited, with each province commonly allocated 5-10 fisheries inspectors. The equipment/vehicles supporting enforcement process are poor and less invested (only 1-2 boats per province for fishery enforcement). Patrol boats are mostly improved fishing vessels with weak engine meanwhile violators' boats are quite speedy and mobile. The field allowance for fisheries inspectors, paid on top of salary for work in the field is quite low (range 10,000-15,000 VND/inspector/day (less than \$1 USD)).

Fines associated with the illegal use of destructive fishing methods, declared in 2005, are detailed in Table 1. Despite these fines,

cyanide fishing remains a widespread problem in Vietnam, where the laws have been difficult to enforce (Burke et al., 2002), as is the case elsewhere in south-east Asia.

The fines related to destructive fishing are not in line with the authority of inspectors and are impractical to apply in the field. The lowest fine likely to be applied to cyanide fishing is 3 million VND but at the local/district level fishery inspectors are limited to issue a maximum fine of 200,000 VND (Article 38 of Ordinance 44/2002/PL-UBTVQH10). That means that only the provincial level inspectors, engaged specifically in management and political field, are capable of issuing the full scope of fines. Inspectors in some cases do not follow the correct rate of fines or procedures in fining. The fines and cases may also not be reported.

There is a lack of awareness of fishers who largely have a poor education, pressures from increasing subsistence and poor access to alternative livelihoods.

During dialogues with community members in MCD's project sites in the Red River Delta

Table 1: Fines associated with illegal destructive fishing practises in Vietnam promulgated in 2005

Violation	Fine (VND)	Fine (USD)	Article*
Use of a net with mesh smaller than regulation	200,000 - 300,000	6 – 20	10.2(b)
Chemical fishing (or storing chemicals onboard a fishing vessel)	3,000,000 - 5,000,000	200 - 300	10.5(a)
Electro fishing	1,000,000 - 6,000,000 depending on engine capacity	60 - 375	10.6(a) – (c)
Explosive fishing (or storing explosives on board a fishing vessel)	5,000,000 - 10,000,000	300 - 625	10.9
Additional sanctions include the confiscation of gear, catch and the destruction of equipment.			11

* Decree No. 128/2005ND-CP of October 11, 2005, Providing for sanctioning of administrative violations in the fisheries domain.

and Central Vietnam it was revealed that deterrents to using illegal destructive fishing methods were insufficient due to:

1. The fines being so low that even if the equipment was confiscated it was still profitable for the fishers to buy new equipment and continue fishing.
2. Enforcement patrols were very rare.

During 1998-2001, the National Directorate of Aquatic Resources Exploitation and Protection (NADAREP) dealt with 355 cases of blast fishing, 106 cases of chemical fishing and confiscated 846 liters of chemical solution (Vu Huy Thu, 2001). Particularly in 2007, dynamite fishing reduced, but still occurred in some areas. In 2007, 53 cases were fined and treated (19 cases: dynamite fishing, 34: storing, sale/trade dynamite), keep 361 fuse, 258.2 kg dynamite, 137.8 m slow fired wire. Electro fishing still occurred in some places, fine 991 cases, treat 510 cases, keep 555 battery, 1017 electrify set and 226 illegal tools, fine over 627 million VND (40,000 USD).

Conclusions and practical steps for a way forward

This report highlights the current lack of information on the extent and impact of destructive fishing practises in Vietnam, in particular the use of cyanide. This information could be made available through more dedicated monitoring and research efforts of the government, fisheries managers and research institutes.

As one village leader for central Vietnam was quoted as saying “illegal fishing only benefits one family but destroys the living of 10 others”. Understanding of the extent and impact of destructive fishing practices is critically needed as the basis for issuing legislation to control the situation as well as for complying with and enforcing the rules. In addition, it also enables the fishers to make

sensible choice and trade-off amongst the fishing practices.

The report also confirms that the Government of Vietnam is clearly committed to eliminating the use of destructive fishing methods with a clear domestic legal mechanism as well as commitments in various international fora.

Besides, it draws the attention that low compliance and weak enforcement are the key challenges in management of the destructive fisheries. Although it has been almost 20 years since destructive fishing methods were first made illegal their use is still wide spread. It suggests the key to the success of these commitments will be an effective compliance and enforcement mechanism. The immediate need now is to build capacity and enabling regulations and policies to ensure effective implementation of these national policies and legislation.

It is also implied from the facts provided in the report that the legislative controlling efforts from the government could not be successful if the issue of sustainable livelihoods for fishers who exercise illegal fishing is not effectively handled. Lack of awareness of the long term implications of destructive fishing, lack of knowledge of actual damage caused by cyanide in particular, and no alternative fishing methods or enabling policy for it, are among the problems that need to be addressed.

Having highlighted the above, in this report, key issues and recommendations are also provided (see table 2) focusing on assessing the status of the use of cyanide, evaluating the impact of harvesting and developing and implementing sustainable management. Such issue and recommendations mainly target the attention of the government authorities, where the Centre of Marinelife Conservation and Community Development as the hosting

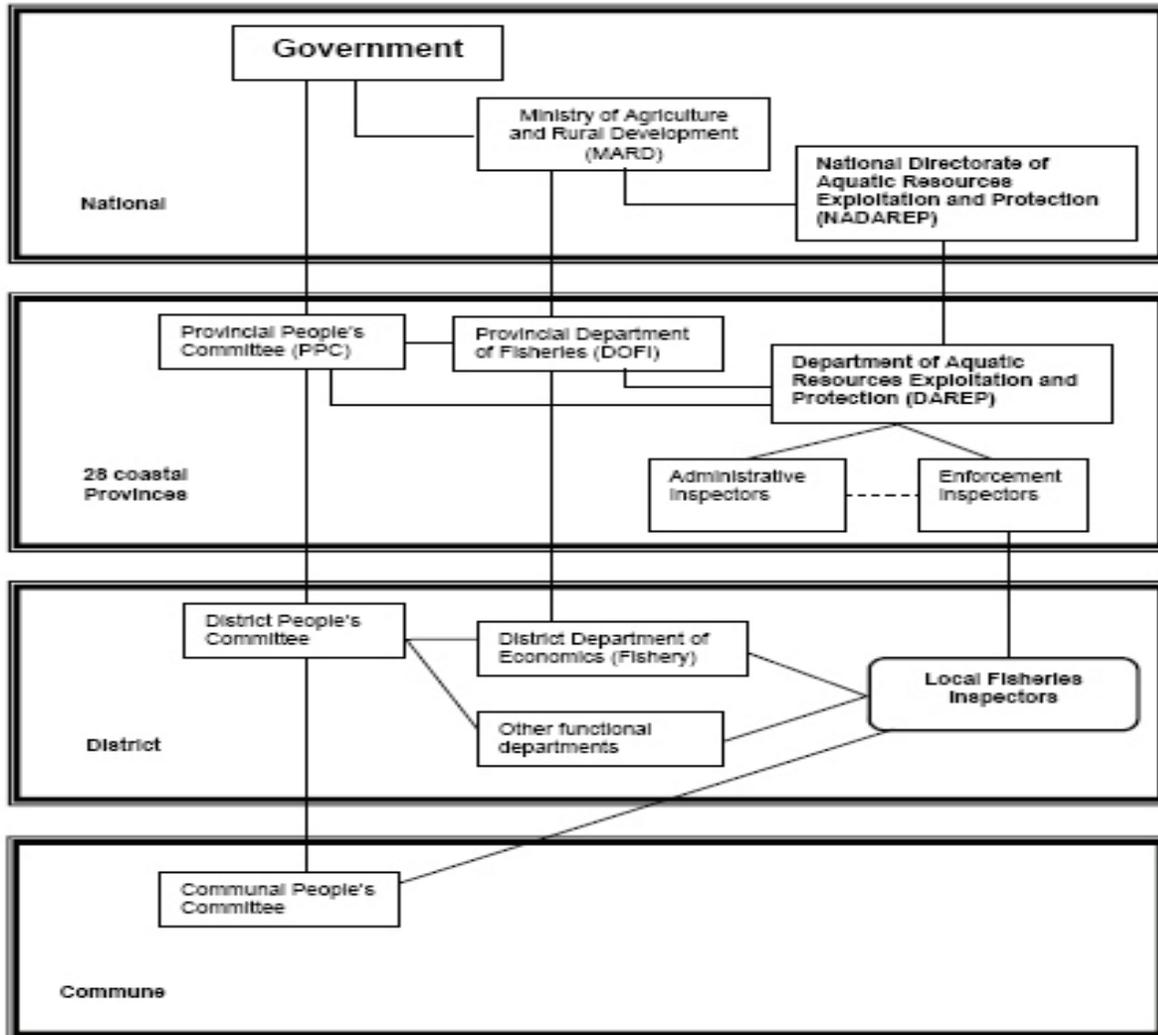


Figure 2: Administrative structure for fisheries enforcement in Vietnam

organization of the report authors is also in line with its vision, mission and fields of recommended to take part in the process, expertise and interest.

Table 2: Issues and recommendations

ISSUES	RECOMMENDATIONS	MCD ACTION
1. Status of cyanide fishing		
Lack of information on the status of destructive fishing in Vietnam, especially cyanide fishing	Determine the extent of cyanide fishing in Vietnam	Support and assist researchers to carry out related studies
2. Enforcement		
Fines not strong enough to prevent violators in the future	Strengthen the awareness of enforcement forces	Support organisation of training useful to enforcement strengthening
Insufficient enforcement resources	Call for international support for capacity building and equipment (vehicles; devices) More investment required from local authority	
Punishment mechanism inadequate	Improve the authority of field enforcement inspectors	
Confusing institutional arrangements	Build a clear mechanism to allocate rights and responsibilities for involved institutions	
A clear understanding of the livelihoods of people fishing destructively is essential to the design of enforcement and patrol	A socio –economic assessment of fishers using destructive methods to be undertaken to inform appropriate management responses	
3. Awareness raising		
Lack of awareness of the long term implications of destructive fishing	Awareness raising of the long term implications of destructive fishing supported by strong scientific proof	Support organisation of community dialogues of destructive fishing impacts
No alternate fishing methods available	Development of alternative sustainable fishing methods	Support poor fishermen to alter their livelihoods
Lack of knowledge of actual damage caused by cyanide	Promote the understanding of serious damage of cyanide to fishermen and managers	Support to organise the trainings and awareness campaigns
Limited cooperation among fisheries inspectors, border military, marine police/security and information-culture force	Awareness raising for dangers of use of cyanide Communication via public media about fisheries law/regulations Develop a cooperative mechanism between stakeholders to strengthen effective enforcement	
4. Improvements for legal framework		
Lack of subsidy policy for enforcement inspectorate	Assess the risk facing the enforcement inspectorate and build an adequate support mechanism	
No institutions, policy for poor fishermen to alter livelihood	Investigate development of local regulations for destructive fishing	Advocate the alternative livelihood needs of small-scale fishers
Low regime for fisheries conservation inspectors	Assess the adequacy of the level of fines for illegal destructive fishing	

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Acronyms

- CDT • Cyanide Detection Test
- DAREP • Department of Aquatic Resources Exploitation and Protection
- DOFI • Department of Fisheries (Provincial Level)
- GoV • Government of Vietnam
- IMER • Institute of Marine Environment and Resources
- MAF • Marine Aquarium Fishery
- MARD • Ministry of Agriculture and Rural Development
- MCD • Centre for Marinelife Conservation and Community Development
- MOFI • Ministry of Fisheries (merged into MARD in 2007)
- MPA • Marine Protected Area
- NADAREP • National Directorate of Aquatic Resources Exploitation and Protection
- PPC • Provincial People’s Committee
- RIMF • Research Institute of Marine Fisheries
- VIFEP • Vietnam Institute of Fisheries and Economic Policy
- WRI • World Resources Institute

Appendix 1: Key international agreements relating to destructive fishing in Vietnam

Agreement/Convention	Detail	Date joined
United Nations Convention on the Law of the Sea (UNCLOS) (1982)	Articles 61 through 64 ,116-119 and 123 – promoting responsible fishing practices	1994
Convention on Biological Diversity (CBD)	Contains articles concerning conservation and sustainable use of biodiversity components as well as the equitable sharing of the benefits and relevant technologies application.	1994
FAO Code of Conduct for Responsible Fisheries (1995)	Contains a commitment to end destructive fishing practices	1995
Basel convention	Control of transboundary movements of hazardous wastes and their disposal Cyanide can be consider a hazardous waste from industrial production	1995
APEC Fisheries Working Group	Endorsed a resolution urging “all members of the international community to cease the practise of cyanide fishing”	1997
World Summit on Sustainable Development (2002)	Contains a commitment to phasing out destructive fishing practices in the marine environment by the year 2012 in accordance with chapter 17 of Agenda 21	2002
Putrajaya Declaration of Regional Cooperation for the Sustainable Development of the Seas of East Asia	Signed among 12 littoral states, recognizing the importance of sustainable development and management of coastal and marine resources within the region, and committing individual and collective efforts of the countries to the implementation of the Sustainable Development Strategy of the Seas of East Asia (SDS-SEA). It includes developing measures against destructive fishing practises Objective 3 s.3(b). www.pemsea.org/pdf-documents/sds-sea/SDSSEA-Full.pdf	2003

Appendix 2: Key domestic legislation relating to destructive fishing in Vietnam

Law/Ordinance/Decree /Decision	Title	Detail	Date Signed
Ordinance 18-HDNN	Ordinance on the protection and development of aquatic resources Promulgator: Government council, signed by VN president	Chapter II, Article 8 (1) Bans the use of poison, dynamite, electro which destroy, kill aquatic resource for aquatic resource exploitation	1989
Directive 01/1998/CT-TTg	Directive strictly banning the use of explosives, electric impulses and toxic to exploit aquatic resources Promulgator: The Government	Prime Minister's instruction on prohibiting the use of dynamite, electric shock and poisons to extract fishery resources	1998
Decision 1971/1999/QD-BCNMT	Decision promulgating technology process to demolish or re-use cyanide Promulgator: Ministry of Science, Technology and Environment	Technology process of cyanide demolition or re-use. The Decision declares that cyanhydric acid and its cyanide salts are critically powerful poisons, one person can be killed with only about 50 mg.	1999
7/2003/QH11	Fishery Law Promulgator: National Assembly	Article 6 (6) Prohibits fishing activities include the production, circulation and use of prohibited fishing gear; the use of prohibited fishery activities and methods; the use of explosives, poisons, electric and other destructive fishing methods.	2003
Decree 109/2003/ND-CP	Conservation and sustainable development of wetlands Promulgator: The Government	Article 7 (4) Prohibits activities including the use of serially destructive catching methods such as electricity shock, blast, chemical, toxic matters, various nets with mesh size against regulation to fishing in wetlands.	2003
Decree 121/2004/ND-CP	Providing for sanctioning of administrative violations in the environment protection domain Promulgator: The Government	Article 21 (3) Fines of 20 to 30 million VND are applied to activities letting out and excrete into environment pollutants excessive to allowable environment standard. Cyanide is not mentioned specifically but pollutants generally.	2004

Law/Ordinance/Decree /Decision	Title	Detail	Date Signed
52/2005/QH11	Environment Protection Law Promulgator: National Assembly	Article 7 (2) Strictly forbids activities including exploitation and capture of biological resources by destructive tools and methods, and opposed to season and production allowed by regulations.	2005
Decree 128/2005/ND-CP	Providing for sanctioning of administrative violations in the fisheries domain. Promulgator: The Government	Provides fines for electro, dynamite and chemical fishing (see table 1 for details)	2005
Decree 12/2006/ND-CP	Detail rules to implement Trade Law in international goods business and agency practices on trading, processing and transiting to foreign countries. Promulgator: The Government	Appendix 03 (VII) Ministry of Industrial manages the export and import of toxic chemicals and products in the list of allocated goods.	2006
06/2007/QH12	Chemical Law Promulgator: National Assembly	Article 7 (4) Prohibited chemical practices include the use of toxic matters to hunt animals and the implementation of activities detrimental to human health, property and environment Cyanide is not mentioned specifically.	2007
Decision 79/2007/QD-TTg	The National Action Plan on Biodiversity up to 2010 and orientations towards 2020 for implementation of the Convention on Biological Diversity and the Cartagena protocol on Biosafety Promulgator: Prime Minister	Article 4 (c) Prevention, control and strict handling of illegal exploitation, trading and use of biological natural resources to eliminate destructive methods of exploiting biological natural resources and the destruction of sensitive ecosystems.	2007

Summary of the Use of Cyanide in the Philippines

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Background

Live reef fish trade started as a hobby in early 1950s, based on BFAR studies and notes. Trade picked up after 10 years as other business men began investing. Importance of the LRF trade was noticed only in 1968 when export reached 3,931kg amounting to PhP 153, 329.00 (quite substantial at that time). In 1970, export value reached a million pesos and increased twenty-fold in a span of ten years. In the subsequent years, the live reef fishes are within the top ten fishery products exported by the country.

Traditional methods include fine mesh nets, modified spear guns and hookah diving. Due to the lucrative nature of this business, catchers opted for easy, less expensive and environmentally destructive techniques such as the use of cyanide to stun the fish.

Under Philippine laws, the trade of cyanide tainted live reef fish is not allowed. Such laws are Republic Act 8550, "Fisheries Act of 1998"; and Republic Act 6969, "An Act to Control Toxic Substances, Hazardous and Nuclear Wastes". RA 8550 - Section 88 prohibits the use of obnoxious substances e.g. cyanide in fishing. Under this law, the Bureau of Fisheries and Aquatic Resources is mandated to develop, improve, manage and conserve the country's fisheries and aquatic resources. BFAR monitors and regulates the importation and exportation of fish and marine fishery and aquatic products.

RA 6969 specifies the requirements pertaining to the importation, manufacturing and use

of cyanide and cyanide compounds and its storage, transport and disposal of its generated wastes, and provides penalties for violations and for other purposes. The Department of Environment and Natural Resources (DENR) is the lead agency in the regulation of registration and importation of chemicals. BFAR as a cooperating agency issues endorsement letter provided that the client met the requirements.

Cyanide Detection Test

Before the 1990's, cyanide analyses were conducted at the Chemistry Section of BFAR's Post Harvest Division. The method used then, Picrate Method, can only give qualitative results, e.g. positive or negative. In the 1990s the distillation and ISE analytical method was introduced.

CDT labs serve two major functions. The first is support to law enforcement activities. Such activities may include evidences that require laboratory tests like fish and substances suspected to contain cyanide like the tablets and water in squirt bottles. When needed the laboratory chemist may be called to court hearings as an expert witness. The second is required by law that is analysis of samples for trading purposes, i.e. laboratory testing for live fish prior to shipment. CDT laboratories analyzed 2800 (on the average) samples in a year with the majority coming from the Palawan area.

BFAR contracted the operation of its CDT labs with IMA since the agency was undermanned to attend to the large volume

of samples required for analysis. Under this contract several of the laboratories were established. There were six laboratories established and these were located in the strategic areas of Palawan, Leyte, Davao, Cebu, Zamboanga and Manila.

In 2001, BFAR regular employees operated the CDT labs when contract with IMA terminated. Due to logistics constraints, only four laboratories remained operational and these are the Puerto Prinsesa, Cebu, Zamboaga and Manila. However, at a later date, BFAR was able to acquire patrol vessels equipped with several laboratory equipment including CDT that can be relocated to land-based laboratories.

Methods. Cyanide is recovered from fish samples through distillation. ISE method is used to quantify the cyanide in the sample. Results are reported as mg CN/ Kg meat. To ensure the reliability of results, the laboratory protocols adhere to standard operating procedures (SOPs). These SOPs are:

- Calibration – using standard cyanide solutions, being the basis of quantifying cyanide content is done at the start of every analysis
- Checking of linear response – conducted together with calibration since quantitative determination is done using a linear calibration curve
- Distillation of water and fish tissue matrices spiked with cyanide is conducted to check cyanide recovery
- Electrode efficiency is regularly checked according to manufacturer specifications

Procedures. There are several collection points wherein samples can be collected. These are fishing boats, holding tanks, and cages. The required number of samples are

collected at random based on the number of fishes to be shipped out. Submission of samples require accompanying documents, which are:

- Collection and submission form which contain information directly relating to the sample. This information includes the names and addresses of the fishermen, date and place of collection etc. Information that accompanies the sample will enable authorities to trace and link it to the fishermen/trader, especially for legal purposes
- For Palawan in particular, there is the Certificate Receipt of Live Fish Taken for Examination which contained information on the origin of the sample as certified by designated authorities
- If applicable, apprehension reports are also provided to the laboratory for added information

Issues:

Accessibility. The first issue is the Archipelagic nature of the Philippines. Some areas have difficult access to the laboratories due to the travel time involved, especially those coming from island areas.

From the laboratory point of view, this archipelagic nature poses problems first in the purchase of supplies, particularly chemicals, which have specific requirements during transport. The second is the need for repair and preventive maintenance conducted by product engineers. In both cases, suppliers and their respective engineers are located in key cities like Manila and Cebu. One way to mitigate this problem is to have spare sets of equipment and a relatively larger stock of supplies which will require more financial expenses.

Due to the island nature of the Philippines, some samples are sent via couriers or representatives which poses sample security concerns. With commercial couriers, signs of tampering can be easily checked; however, with samples sent via representatives (in kind), which could be unauthorized, there is a need to come up with procedures to prevent tampering.

Lack of Manpower. When BFAR took over the operation of the CDT labs and its associated responsibilities and tasks, these were to be performed on top of the existing responsibilities. These activities include inspection and sampling at the start of supply chain, laboratory and administrative work. With all the other activities, BFAR personnel are faced with multi-tasking. Laboratory analysts are tasked not only with cyanide analysis but also red tides, chemical, microbiological and antibiotic residue analyses. In addition there are no sufficient vacant positions in the government to absorb those that came from the IMA-run laboratories.

Unlike when the labs were under the management of IMA, there are personnel for each specific task. These tasks are for chemists and biologists, and hiring personnel with these qualifications is also hampered by the lack of vacant positions, or, as in the case of services charged to maintenance and operations cost, the salary allowed is not commensurate. Sometimes there are those who accept the position but do not stay for very long. This results in a high turn over rate, which is not beneficial for the laboratory.

Most regulatory officers are not properly informed on how to collect samples. Copies of protocols or “how to’s” were sent to the regional labs. Most often the protocols were followed on samples sent to the labs early on. But later, there were instances wherein

the proper protocols were not followed, compromising the integrity of the samples and leaving the lab with no alternative but to issue rejection. The regional officers/staff had to be regularly reminded of the protocols.

Final inspection of live fish is at the port of exit. Fishery quarantine personnel may not be properly informed when to look for CDT results in shipments of fishery products.

What Is Needed:

Expansion/Additional labs. With the above mentioned issues, if BFAR wants to expand its CDT activities several things will be needed. There is a need for additional laboratories in several areas in order to have easier access to its services.

It is suggested that several labs be established in Palawan due to the live fish trade present in almost all of its municipalities. Other areas were chosen so that the regional counterparts will have a more direct access to the lab.

Capacity building. Training of personnel, especially for law enforcers (PNP Maritime, Coast Guard, Local Government Units, BFAR Regional Offices), is required especially for sample collection and sample handling in order to assure that samples will arrive at the laboratory at a viable state. Moreover, management training is necessary for people at the local level to further enhance capabilities policy formulation.

Environmental awareness. There is a need to conduct environmental awareness training for local fisher folk to emphasize the negative long term effect of destructive fishing techniques to the environment including fish habitats.

Field test kit. BFAR recognizes the inadequacies of the current method employed and more importantly the need for cyanide testing for regulatory purposes. BFAR is open to collaborative work in particularly in the investigations of methods for cyanide detection. In the past, BFAR, MAC and Merck jointly conducted a comparative study between a methods developed by Merck. The development of a method particularly ideal for field use will be beneficial to BFAR.

Alternative livelihood for displaced fishermen. Possible alternative sources of income are necessary to wean fishers from cyanide fishing. Fishermen resort to this destructive fishing method due to a complex set of issues, i.e. poverty, easy cash returns at less capital, etc.

Sustainable Financing

Maintenance & operation per lab per annum

Chemicals	200,000.00
Laboratory supplies	100,000.00
Office supplies	30,000.00
Labor Services (1 chemist, 2 lab assistants)	500,000.00
Utility bills/expenses	125,000.00
Total:	PhP 955,000.00
	US\$ 23,507.11*

(*US\$1.00 = PhP 40.626 01 Feb 08 exchange rate, www.bsp.gov.ph)

Finances

In the establishment of the laboratory:

New structure:

Per Laboratory	900,000.00
If structure is to be rented	
Renovation (fit for lab)	100,000.00
Rent (Manila rate) per month	75,000.00
Conduct of nationwide training (regulatory and fisherfolk)	1, 000, 000.00
Production of IEC materials	500, 000.00
Total	PhP 1, 500, 000.00

Request for Assistance in the Operation of an Expanded Cyanide Detection Test (CDT) Laboratories in the Philippines

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Background

While the misuse of cyanide in live fish collection started in the Philippines in the early 1950s, the country was also the first to implement a comprehensive Cyanide Fishing Reform Program anchored on the adoption of the world's first-ever technology for the detection of the presence of cyanide in fish tissues and organs.

Between 1995 and 2001, the Philippine Bureau of Fisheries and Aquatic Resources, using funds sourced from a Fisheries Sector Program loan, set up a nationwide network of laboratories, acquired the necessary equipment and chemicals, hired staff, organized mobile monitoring, control and surveillance (MCS) teams and set in motion an integrated reform-oriented program dedicated to combating the cyanide fishing menace.

During that period, thousands of fish were tested for the presence or absence of cyanide, several live fish shipments were confiscated, hundreds of fishermen were arrested, a few dozens of whom were successfully prosecuted in court.

The original CDT laboratories were located in: Metro Manila; Puerto Princesa in Palawan; Cebu; Davao; Tacloban; and Zamboanga.

They served two major functions:

First was to respond to request from law enforcement agencies for evidentiary support against cyanide fishing violators.

The second was to satisfy the requirements of a local law in the Province of Palawan which required CDT laboratory testing for live fish shipments. In Palawan, the only live fish that could be sold and transshipped were those that had been certified cyanide-free.

Unfortunately, shortage of funds compelled BFAR to scale down the operation of the laboratories. Faced with a budget that had remained stagnant for years and instructed by the Department of Agriculture to use limited resources in production-enhancement activities, BFAR was compelled to integrate the functions of the CDT laboratories with the activities of BFAR's central office laboratory whose many responsibilities include red tide monitoring; chemical, microbiological and antibiotic residue analyses; fish health, and fishery product quality control management.

To date, only four of the six laboratories are functioning. These are the ones located in Manila, Puerto Princesa, Cebu and Zamboanga. However, only the laboratory in Puerto Princesa is operating full-time and this is largely because the city government is subsidizing its operation.

Political Will

Having planted the seed of reform in the live coral reef fishery, BFAR finds it frustrating that a prolonged financial drought has stymied the momentum it has painstakingly built towards combating cyanide fishing.

Resourcefulness has enabled our agency to acquire complete laboratory equipment for seven more laboratories. Unfortunately, our optimism about being able to raise funds to set up and operate at full capacity an expanded CDT network has faded with the onset of discouraging developments in the financial front.

It is extremely disappointing that while BFAR's resolve to stamp out cyanide fishing remains strong and unwavering, there is not enough money to get our agenda of reform off and running.

Conviction

BFAR is convinced that no effort to regulate, manage and reform the marine ornamental trade will succeed unless it is supported by a solid enforcement tool in the form of a credible cyanide detection testing technology. We believe that the ISE method we have been using since 1995 works. We have no doubt that this technology will remain a strong deterrent

to cyanide misuse in live fish collection especially in provinces and municipalities where the problem is prevalent.

Needs and Wants

BFAR is hoping for financial assistance in the form of a long-term grant that will enable us to re-invigorate our anti-cyanide fishing campaign. As we already have seven brand-new complete CDT equipment on top of the six existing ones that may need minor refurbishing, BFAR has the potential to set in motion an expanded network of 13 laboratories strategically located in major centers of ornamental fish collection. But we need operating funds to be able to hire project management staff and consultants, pay rent, procure chemicals and supplies, acquire on-shore and off-shore mobility, conduct monitoring, control and surveillance (MCS), support enforcement activities, undertake information and education activities and provide skills enhancement trainings to project staff.

BFAR is also open to collaborative work in the development of a simpler – and, if possible, portable - CDT test. The development of a method particularly ideal for field use will be beneficial to BFAR given the country's archipelagic character.

Legal and Management Measures towards Sustainable and Community-based Marine Ornamental and Food Fishery in Indonesia

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Introduction

The past 15-20 years have witnessed a progressive deterioration in Indonesia's coral reefs. A regional survey by Indonesia's Academy of Sciences (LIPI) found that only 6.7% of Indonesian reefs are still in excellent condition. Overfishing and destructive harvest techniques negatively influence biodiversity in all coastal and marine ecosystems, including mangrove forests, inter-tidal areas, sea grass beds, coral reef systems, and the pelagic and abyssal zones. The widespread use of cyanide fishing to collect marine ornamental fishes and live reef food fishes are well-documented in the Indonesian archipelago, as are the resulting lethal effects upon the marine environment.

The global trade in marine ornamental fish and associated accessories is an industry in excess of USD 70 million per year. Indonesia is one of the world's leading exporters of products derived from coral reefs, and the overwhelming majority of these are collected in unsustainable ways. Millions of people worldwide, in Asia and in Indonesia depend on coral reefs, and unsustainable harvesting

techniques jeopardize livelihoods of coastal communities.

The trade in marine ornamental fishes and accessories first took root in Indonesia in the 1960s. In the early 1990s Bali became the major base for the marine aquarium industry in Indonesia, replacing Jakarta and Surabaya. From 1990-1999 total export volume of reef fishes from Bali grew from 2.14-3.94 million reef fish per year; with peak levels reached in 1996 when 9.78 million reef fish were exported.

It is interesting to note that though export volume peaked in 1996 and thereafter slowed, it declined further with the onset of the Asian financial crisis in 1998. The price setting power in the marine ornamental and live reef food fish industry lays solely in the hands of the exporters who ship internationally from Bali, Surabaya, Manado, and Jakarta. Yet the crisis was not the only factor which influenced export volume from Bali after 1996. Internal political chaos in Indonesia starting from 1998 led to an unhealthy business situation whose repercussions were experienced most

gravely by fishing communities. The financial and political crisis is not the only factor that contributed to a decrease in export; reef degradation caused by blast fishing and cyanide fishing may also have contributed to a decrease in export of aquarium fish from Indonesia.

Action Plan Against Cyanide Fishing

The Ministry of Marine Affairs and Fisheries of the Republic of Indonesia (MMAF), in collaboration with Telapak and other national and international organizations have been developing an action plan to abate cyanide fishing. The action plan includes the following components:

Institutional Set Up and Capacity Building

MMAF is the National Management Authority for fisheries, coastal and marine affairs. Some of the authorities related to coral reef and protection of endangered species are now being transferred from the previous authority, the Ministry of Forestry, to MMAF. When the transfer of authority is completed, MMAF will have full authority and responsibility to enforce CITES for coral reef organisms and other aquatic species in the list. The ornamental fish industry has so far been very loosely regulated by MMAF. For instance, to export ornamental fish the exporter will only need two documents: a Health Certificate issued by the Immigration and Customs Agency, and an Export Notification issued by the Immigration and Customs Agency in coordination with the Ministry of Trade. For fishes and coral reef species under CITES, it was the Ministry of Forestry which had been the agency to issue regulations and permits. It is envisaged that a stronger MMAF will be able to regulate ornamental fish and coral reef exports as the National Management Authority.

The MMAF plans to establish Technical Managements Unit at the regional level, and MMAF will also explore the establishment of district-level Technical Management Units at districts that participate in COREMAP Phase II. These units will be tasked with implementation of regulations related to fisheries, coastal and marine affairs. Initially, these units will be established at main fisheries centers, including Bali, Jakarta, Sorong, and Manado. It is envisioned that Coral Detection Testing Labs will be under these regional Technical Management Units, and MMAF will explore the possibility to contract out the establishment and management of the labs to NGOs or outside contractors. Under the World Bank / ADB Coral Reef Rehabilitation and Management Program Phase II, tools and technologies for cyanide testing will be explored.

The MMAF will soon have the capacity to enforce law on fisheries. The Directorate-General of Surveillance and Control of Marine Resources and Fisheries, MMAF has authority to conduct investigation and prosecution of violations of fisheries-related laws. The capacity development plan of this Agency includes recruitment of personnel, trainings, and procurement of equipment. Trained staff of this Agency will have the right to bear arms.

Under the existing law, violations against fisheries related laws are to be prosecuted at Fisheries Tribunal, which initially are present in Jakarta, Medan in North Sumatra, Pontianak in West Kalimantan, Bitung in North Sulawesi, and Tual in Molucca.

Capacity building for MMAF to be able to take on these new authority and responsibilities will include strengthening of human resources, both in terms of number of personnel and quality of those personnels,

and securing sufficient operational funds for the establishment and running of labs for detection of cyanide as well as other chemicals that are prohibited for catching fish.

The physical infrastructure needed for these policy direction of MMAF will include establishment of labs at regional/export centers, and provision of fast, easy, cheap, and portable Cyanide Detection System at the District Level, to be operated by a District agency.

Legal and Policy Framework

The MMAF will employ a two layer approach in reforming cyanide fishing in Indonesia. The first layer will be setting up a legality standard, which will be the first step towards the second layer: ecolabel certification. The legality standard will be applied through licensing and permits based on existing regulations, including the Fisheries Act No. 31/2004 which explicitly prohibits the industry from using chemical substances and pose sanction up to 10 years imprisonment and a fine of maximum Rp 2 billion. The Fishery Resources Conservation Decree No. 60/2007 regulates that "...fish harvest must hold a license..." and "...licensing considers fishing techniques and gear...". It is envisioned that MMAF will issue a Ministerial Regulation and Decrees which will include a cyanide-free certificate as a requirement for the legality of marine ornamental business and export. For coral reef trade, the existing regulations call for culture permit, trade permit, and a quota system for export. This regime is currently under the Ministry of Forestry, soon to be transferred to MMAF. This will also become the legality requirement for companies to meet the legality standard regarding coral reef trade.

When the legality standard has been developed and implemented, the development of a

certification system for marine products will be possible. Learning from the success of certification system in forestry, and failure of existing certification system for marine product as developed by the Marine Aquarium Council (MAC), the development of an ecolabel certification system for marine product should consider the following: 1. Development of economic, social, and environmental criteria, 2. A national processes which shall be based on multi-stakeholder constituency, involving meaningful participation and ownership of MMAF, the industry, NGOs, and fisherfolk community, and 3. Market education and reform.

Telapak's Integrated Approach to Destructive Fishing Reform

While MMAF has been developing an institutional arrangement and legal and policy framework in abating cyanide fishing, Telapak is contributing to the goals of sustainability and people-centered marine ornamental industry through an integrated approach to destructive fishing reform. This calls for an integration of conservation purposes and improvement of local livelihoods. As have been implemented in the villages in Bali, the intervention into destructive fishing includes: trainings the fisherfolk in environmentally fishing techniques, transfer of science and technology from universities and the high end industry to be adapted into local cost and material, building local organizations and institutions, and developing micro-enterprises to reflect the "from fisher to exporter" changes, and public education.

Reforms of the marine ornamental industry in Indonesia will call for transparent and independent monitoring system based on the networks of civil society as its constituent. Under this direction, Telapak is working with other NGOs and research institutions to develop Marine Watch Indonesia. This should

become the partner and counterpart of the CDT labs network especially, the marine ornamental industry reform in general.

In 1997, Telapak collaborated with the IMA to implement the Destructive Fishing Reform Program (DFRP) in Indonesia. Cyanide testing was one component of the DFRP; a broader community-based program involving rapid resource appraisal, net-training and other alternative livelihood training programs, enterprise trainings, marine education of school children, instruction of fisherfolk in coastal conservation practices, farming corals and giant clams, and mapping using geographic information systems (GIS). Plans by Telapak to implement CDT in Indonesia in collaboration with the IMA (discussed at the International Coral Reef Symposium held in Denpasar in 2000) did not happen because of the lack of funding and other problems.

Telapak's main achievement with destructive fishing reform was the establishment of two villages, which are presently completely cyanide-free. Through education, economic incentives, and peer pressure, the villages now capture and export totally cyanide-free MAF from the village of Les (situated in northern Bali) and farm artificial live rock and corals grown from fragments in the village of Serangan (situated near Denpasar in southern Bali). Telapak in collaboration with the Philippines based East Asia Seas and Terrestrial Initiatives have succeeded in changing the behavior of fisherfolk for sustainable use of marine resources and the implementation of reef restoration programs. This was achieved after a number of setbacks. Community organizing, technical intervention, management support, market reform, and educational components were integrated into a holistic community-based program supporting conservation of marine ecosystems and sustainable use by the MAF fishery.

WORKSHOP ABSTRACTS

Ion-Selective Electrodes for Cyanide Detection: Present Status and Future Possible Improvements

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We all want a cyanide analytical technique that is as simple and fast as possible, inexpensive, capable of being done by minimally-trained personnel; that will withstand judicial scrutiny. Ultimately, it may be possible to meet all of these requirements, but not within any short-term time frame. Cyanide in fish, as in other animals, is rarely present in the unbound form, and requires pretreatment to free it up for measurement. After digestion and distillation, the sample consists of unbound cyanide ion in an alkaline background and, from the analyst's point of view, is the same whether it originated in fish tissue or wastewater.

Round robin tests on cyanide "unknowns" were conducted at different times by the two recognized volunteer U.S. standards organizations, using a method with a carefully spelled-out distillation step and cyanide ion-selective electrode (ISE) measurements. Both tests had extremely good results,

including lower limits of detection on a linear calibration plot to cyanide levels of 0.03 and 0.06 ppm. A more recent EPA-funded third party evaluation had similar results. The cyanide ion detection method, involving acid digestion, distillation, and ISE measurement, is approved by the U.S. Environmental Protection Agency (EPA). This information is all in the literature, and should give strong backing against any legal challenges.

There are a number of interesting options for modifying the present method, and these will be discussed. They include techniques for electrode measurements down to 0.003 ppm, several possible changes in the distillation step, and the use of other electrodes, such as the silver ISE or an ISE for HCN. The problem is that validating any changes will require extensive testing, independent collaborative data, and field experience before they can be used for enforcement.

Cyanide Detection Testing (CDT) Used by the BFAR/IMA Laboratories in the Philippines

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The use of cyanide to capture marine aquarium fish and food fish alive is widespread in the Asia-Pacific region and not only leads to high mortality rates of the captured fish, but also damages and kills corals and other organisms on the reefs. The use of cyanide is illegal in all countries where it is used, but enforcement is difficult.

A cyanide detection test (CDT) developed by the American Society of Testing and Materials (ASTM) was adopted by the International Marineline Alliance (IMA) and applied to test marine organisms (mostly aquarium fish and food fish species) in the Philippines under contract with the Philippine Bureau of Fisheries and Aquatic Resources (BFAR) (APHA 1992, ASTM 1997). A network of six BFAR/IMA CDT laboratories was established throughout the Philippines staffed by chemists. Sampling was conducted by biologists deputized by BFAR at various locations including the boats of fishers, home ports, distribution points such as airports, and at export facilities in Manila. Over 48,000 fish and invertebrate specimens were tested

for the presence of cyanide by IMA chemists from 1993 to 2001. The method involves the digestion of fish tissues in sulfuric acid to liberate hydrogen cyanide gas, and capture of cyanide ions in sodium hydroxide solution after reflux distillation. The chemicals added to the apparatus facilitate the extraction of cyanide from fish tissues and help to eliminate interfering substances. Cyanide ion concentrations were determined using an ion selective electrode (ISE) linked to an ISE meter manufactured by Thermo-Orion.

The ASTM ISE method for cyanide ion detection is internationally recognized. It has been in use since the early 1980s and is periodically updated in separate publications by the ASTM, the American Public Health Association (APHA), and the U.S. Environmental Protection Agency (US-EPA). The ASTM ISE method has been used by a variety of organizations. Cyanide testing should be used to enforce laws against illegal fishing and to accredit marine ornamentals (MO) as being cyanide-free to support a sustainable MO trade.

References

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Search for a Simplified Field Test: the Soundararajan Digestion Combined with ISE or Colorimetric Methods for Detection of Cyanide Ion Concentrations in Marine Fish

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A new method for testing fish samples for the presence of cyanide was developed by Dr. Rengarajan Soundararajan in 1990 (Rubec and Soundararajan 1991). The method involves the digestion of fish tissues in concentrated sodium hydroxide (NaOH). The method is appealing since it is relatively simple and quick to conduct. The cyanide ion is released from the tissues into a basic, high pH solution preventing the cyanide from being lost to the atmosphere. Cyanide ion concentrations can then be measured with an ion-selective electrode (ISE).

Five fish species obtained from Indonesia tested in the USA had cyanide ion concentrations ranging from 5.8 to 23 mg/kg (ppm) (Rubec and Soundararajan 1991). A Clown Triggerfish obtained from the Philippines exhibited a cyanide ion measurement of 1120 mg/kg. No cyanide was detected in two Flame Angelfish obtained from the Marshall Islands, for two French Angelfish from the Caribbean, and two species of surgeonfish obtained from Hawaii. Hence, cyanide was detected in fish from countries known to have collectors using cyanide and no cyanide was detected in fish obtained from countries where cyanide is not used for collecting marine aquarium fish.

The International Marineline Alliance (IMA) evaluated the technique in 1991, but abandoned it after it was found to give anomalously high cyanide readings. It was suspected that the anomalously high readings found by the IMA might have occurred because of false-positive readings caused by sulfide interference with the ISE electrode.

Further research was conducted by Aquarium Systems with a small grant obtained by the IMA from the Columbus Zoo (Frakes and Studt 1996). The basic procedure was to expose the fish to known concentration of cyanide ion, kill the fish, then puree the samples in 5M-NaOH. The NaOH volume was calculated to yield a 10% by weight fish slurry. This slurry was allowed to settle and a clear aliquot was diluted with distilled water to produce another 10% by weight dilution. Cyanide ion (CN⁻) concentrations in the final solution, 1% fish tissue, and approximately 0.5M-NaOH were measured in millivolts (mV) recorded with an Orion CN⁻ ISE linked to an Orion ISE meter.

These readings were compared with a semi-log plot produced with known cyanide concentrations of 0.1, 1.0, and 10 mg/L

levels with mV readings recorded as each level (Frakes and Studt 1996). Lead carbonate was added to the supernatant solution to precipitate sulfides from solution. The first trial compared readings with Atlantic Blennies, which were net-caught and exposed to cyanide. Both the test and the control fish (not exposed to cyanide) were found to exhibit cyanide levels of about 300 mg/L (ppm). Hence, the addition of lead carbonate to the solution prior to ISE testing did not eliminate the anomalously high readings. A second experiment evaluated whether the high readings might be due to iodide interference with the ISE. Again, the experiment was inconclusive. In a third experiment, test fish (4 Caribbean Blue Chromis, 1 Atlantic Wrasse, and 3 goldfish) were exposed to cyanide concentrations ranging from 1.3 to 3.9 ppm CN⁻ for times ranging from one minute (for the 4 chromis) to 14 minutes (for a goldfish). Readings for control fish (1 wrasse and 2 goldfish) not exposed to cyanide produced higher mV readings than fish exposed to cyanide.

Recent analyses by Ms Benita Manipula using the Soundararajan procedure and cyanide measurements obtained using an Industrial Test Systems (ITS) Inc. colorimetric Cyanide ReagentStrip™ (Cyanide Test Kit 484003) have also experienced difficulty in obtaining reliable measurements of cyanide concentrations in comparison to known concentrations of cyanide ion in sodium hydroxide solution (lacking slurry). The problem does not appear to be related to the reliability of the

ITS cyanide test strips, since they have been demonstrated to be sensitive (down to 0.02 mg/L) and reliable for measuring cyanide ion concentrations in water (Battelle 2005).

The most likely explanation for these results is that organics in the digested tissue solutions containing sodium hydroxide produced the anomalous readings both with the ISE and the ITS test strips. Further research is needed to see whether it is possible to measure cyanide ion concentrations in solutions obtained from marine fish using the Soundararajan tissue digestion method.

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Battling the Silent Killer of Coral Reefs

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The misuse of sodium cyanide - the silent killer of coral reefs - in the live reef fish trade remains a major and fundamental challenge to fisheries resource management and conservation in the Philippines. Although the practice is illegal and fraught with health risks, live fish collectors continue to take their chances, emboldened by the knowledge that government has very limited capability to apprehend and prosecute them.

The country's response to the cyanide fishing menace was originally focused on training programs that encourage collectors to shift to coral-friendly harvesting techniques. However, the failure of the market to reward them with better returns for their enterprise has been cited as a major disincentive that eventually compels reformed fishermen to revert to cyanide use.

The Philippine campaign against cyanide fishing reached a major turning point when our agency adopted a cyanide detection test that uses Ion Selective Electrodes to detect the presence of cyanide in fish tissues and organs. The new test was regarded as a significant breakthrough, inspiring law enforcers to make arrests and enabling BFAR to file cases

and successfully prosecute a number of them in court.

Unfortunately, a shortage of funds compelled BFAR to scale down the operation of its six CDT laboratories. To date, only four of the six laboratories are functioning: Manila, Puerto Princesa, Cebu and Zamboanga.

The good news is that BFAR's political will to stamp out cyanide use remains strong and has only been put on hold due to budgetary limitations. In fact, BFAR has a standing plan to expand the existing CDT laboratory network by establishing new laboratories in other centers of live fish collection. The better news is that the agency has been able to source from recent grants and loans a total of seven complete CDT equipment which, funds permitting, would be used in strategic priority areas.

BFAR, however, needs help in running and maintaining these labs and other complementary activities. It is our hope that this workshop will result - well, eventually - in providing us with the wherewithal to put in operation a serious and effective cyanide fishing reform program.

Trends Determined by Cyanide Testing on Marine Aquarium Fish in the Philippines

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Cyanide has been demonstrated to kill corals and there is scientific evidence that it contributes to the high delayed mortality of marine fish in the aquarium trade. The International Marinelife Alliance (IMA) conducted cyanide detection testing (CDT) on marine aquarium fish and food fish as well as marine invertebrates in the Philippines. The testing was conducted under contract with the Philippine Bureau of Fisheries and Aquatic Resources (BFAR). A network consisting of six Cyanide Detecting Testing (CDT) laboratories and three regional Marine Inspection and Sampling (MIS) offices were established. The CDT network conducted random sampling of fish from collectors, middlemen, and exporters throughout the Philippines. Samples were also collected by law enforcement personnel and voluntarily submitted by fish exporters to determine whether or not the fish were cyanide-free. Over 48,000 aquarium and food fish specimens were tested from 1993 to 2001. Since then, the ASTM method involving acid digestion, distillation, and determination of cyanide concentrations using ion selective electrodes (ISE) has been conducted by staff associated with BFAR. The CDT laboratories in conjunction with law enforcement efforts have served to deter cyanide fishing in the Philippines. The proportion of marine aquarium fish tested with cyanide present

dropped from 43% in 1996 to 8% in 1999, then rose to 29% in 2000. The data are broken down by family to indicate the proportion of specimens with cyanide present or absent. Cyanide testing needs to be expanded in the Philippines and implemented in Indonesia, Vietnam, and Malaysia to support law enforcement and conserve coral reefs and their associated fisheries. It should be tied to an accreditation system of marine ornamental species exported to other countries including the U.S.A.

Rubec, P.J., Pratt, V.R., McCullough, B., Manipula, B., Alban, J., Espero, T. and Suplido, E. (2003) Trends determined by cyanide testing on marine aquarium fish in the Philippines. In *Marine Ornamental Species: Collection, Culture and Conservation* (Cato, J.C. and Brown, C.L., Eds.) 327-340, Iowa State Press, Ames, IA.

Conservation Policies for Abating Cyanide Fishing in Indonesia

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Strategies towards abating cyanide fishing for aquarium fish

- Support for development of sustainable sources of aquarium fish (environmentally sound capture methods including nets, capture of larval fish)
- Certification of aquarium fish (collaboration with Marine Aquarium fish Council, and others)
- Marine Protected Area networks
- Enforcement

Enforcement – constraints

Standard gear for cyanide fishers: cyanide “kit” with weights for quick discarding of squirt bottles and cyanide tablets if a patrol approaches

- Long marketing chains (many small-scale fishers, various traders, wholesaler, exporter)
- Fisheries police and fisheries court not yet fully operational
- Low expertise, low capacity in legal follow up and collection of evidence in rural areas
- No reliable test for cyanide in live fish (especially if already several weeks in holding facility)

Enforcement – solutions

- Hookah compressor ban (at least two districts)
- Further development of Fisheries Police and Fisheries Court
- Community-based enforcement (COREMAP PokMasWas)
- Mobile mini-CDT lab (in development under COREMAP)

Criteria for a test-kit for use in Indonesia

- Must detect cyanide in live fish ca. 2 weeks after capture
- Simple methodology
- Low cost, Portable
- Test results must be acceptable as evidence for Indonesian court

Cooperation towards abatement of cyanide fishing

- Awareness Training in environmentally friendly fishing techniques, handling, and marketing
- Support certification to build a trust relationship between fishers, traders, and aquarium keepers
- Non-Governmental Organization
- Governmental of Indonesia
 1. Enforcement
 2. Regulations

Implementing agencies:

- Quarantine, Technical Implementation Units of Fisheries Service
- Support establishment of MPA networks, as recommended by the Convention on Biological Diversity and other international forums

Coral Reef Rehabilitation and Management Program

- US\$ 80 million, 2005 - 2010
- De-centralized
- World Bank and Asian Development Bank

- Ministry of Marine Affairs and Fisheries, Ministry of Forestry, and Indonesia Academy of Sciences, local governments
- Seven Districts
- Focus on support for Marine Protected Areas (10% of reef in no-take areas)
- Support for Monitoring, Control, and Surveillance
- (MCS), community-based enforcement (SISWASMAS)
- Support for certification, training in sustainable capture methods
- Development of cyanide test-kit

Policy and Legislative Measures Needed to Enhance Enforcement for Combating Destructive Fishing in Vietnam

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Although illegal, use of destructive methods (cyanide, blast and fine mesh nets, etc.) for fishing is still found common in many places in Vietnam. The presentation will feature an overview of the destructive fishing practices in the country together with an assessment of the legal framework currently governing the issue, from both international and national perspectives. Furthermore, an analysis on the compliance and enforcement of these legal instruments will be provided. Such analysis will set the basis for recommendations to be given on practical steps for a way forward.

APPENDIX

APPENDIX I
Cyanide Detection Workshop
Agenda

6-8 February 2008
Orlando, FL, U.S.A.

Wednesday February 6

5:00 p.m. Workshop Check-in
7:00 Welcome Reception and Dinner

Thursday February 7

8:30	Welcome	<i>Keystone</i>
	<ul style="list-style-type: none">• Introductions• Meeting goals• Agenda review• Discussion guidelines• Introduce Cyanide Expert Panel	
	-Dr. Bob Kobelski, Lead Chemist, CDC	
	-Dr. Brian Logue, Assistant Professor, South Dakota State University	
9:00	Fish Collection, Cyanide Use, Management Issues	<i>Dr. Andy Bruckner</i>
9:30	Review of Point of Collection/Export Issues	
	<ul style="list-style-type: none">• Philippines (<i>Gil Adora</i>)• Indonesia (<i>Agus Dermawan</i>)• Vietnam (<i>Thu Ho</i>)	
10:15	Discussion	<i>All</i>
10:45	Break	
11:00	Review of Point of Import Issues	
	<ul style="list-style-type: none">• <i>Ken Goddard</i>• <i>Vicky Vina</i>• <i>Eddy McKissick</i>• <i>Roy Torres</i>	
11:30	Discussion	
		<i>All</i>
12:00	Application of Cyanide Testing in the Philippines	<i>Dr. Peter Rubec</i>
12:30	Lunch	

1:30	Overview of and Potential Cyanide Testing Methods	
	<ul style="list-style-type: none"> • Application of the IMA method • Ion-selective Electrodes for Cyanide: Present Status and Future Possible Improvements • Enzymatic biosensor method • Use of biomarkers to detect cyanide impacts • Rapid field test procedure • The realm of possibilities for cyanide detection 	<i>Benita Manipula</i> <i>Dr. Martin Frant</i> <i>Dr. Karen Mak</i> <i>Dr. Craig Downs</i> <i>Dr. Peter Rubec</i> <i>Dr. Brian Logue</i>
3:15	Break	
3:30	Discussion: Factors to identify appropriate tests for detection of cyanide in marine fishes	<i>All</i>
5:00	Wrap up and adjourn for the day	
6:30	Dinner	

Friday February 8

8:30	Re-cap of Previous Day and Agenda Review	<i>Dr. Bob Kobelski</i>
8:45	Charge to Breakout Groups	<i>Keystone</i>
8:50	Convene Breakout Group Discussions	<i>All</i>
	<ul style="list-style-type: none"> • Detection Methods • Export Country • Import Country 	
10:30	Break items available	
11:30	Breakout Group Status Report/Interim Summary	<i>All</i>
12:00	Lunch	
1:00	Resume Breakout Group Discussions	
2:45	Break	
3:00	Breakout Group Reports and Recommendations	
3:30	Discussion	<i>All</i>
4:30	Next Steps and Wrap Up	
	<ul style="list-style-type: none"> • Future directions and Closing Thoughts 	<i>Dr. Andy Bruckner</i>

APPENDIX II

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APPENDIX III
U.S. Coral Reef Task Force Meeting
May 4, 2006
Washington, DC

Resolution 15-1: Form a Working Group on Enforcement and Utilize Task Force Expertise to Address the Illegal Use of Cyanide and Other Poisons in International Trade

Responsible Party: USCRTF Steering Committee, International Working Group and Enforcement Representatives. Contacts:

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Issue Statement: There is an urgent need for fast, reliable and convenient detection tests for determining whether cyanide and other poisons have been used in the collection of live coral reef fish entering into international trade. Many Task Force member agencies have expertise in toxicology, biomarkers and forensics that could be applied to this issue. Field-based cyanide detection tests would be extremely useful for local management and enforcement authorities in source countries. Tests that could be reliably used several weeks after exposure would also allow the U.S. to apply the Lacey Act to suspected illegal imports.

Although illegal in most countries, the use of cyanide to capture reef fish alive is widespread, and is driven by the lucrative, growing and largely unregulated international trade in live reef food fish and marine aquarium industry. The U.S. is the number one consumer of live coral, live rock and coral reef fish for the aquarium trade and of coral skeletons and precious corals for curios and jewelry. Previous studies have estimated that most live reef fish entering into international trade and imported into the U.S. are collected with the use of cyanide, and thus are illegal.

Background: Provide a brief history of relevant documents pertaining to the proposal being presented to the SC. Include relevance of EO 13089, Coral Reef Conservation Act of 2000, Coral Reef National Action Plan, Coral Reef Action Strategy, Local Action Strategies, Federal regulations or other pertinent guidance as necessary.

Executive Order #13089 for the Protection of Coral Reefs mandates that the U.S. Coral Reef Task Force "...assess the U.S. role in international trade and protection of coral reef species and implement appropriate strategies and actions to promote conservation and sustainable use of coral reef resources worldwide."

The Coral Reef Action Plan calls for efforts to reduce global threats to coral reefs and to reduce the impacts of international trade in coral reef resources, through exercising global leadership in the international arena, strengthening international conventions, providing assistance and strengthening international research and management.

Assessment of U.S. Role in Trade: Under the leadership of the Department of the Interior (USFWS) and the Department of Justice, the Coral Trade Subgroup of the International Working Group conducted an initial assessment of the role of the U.S. in the international trade of coral and coral reef species in 2000. The analysis found that the U.S. is the number one consumer of live coral, “live rock” and marine reef fish for the aquarium trade and of coral skeletons and precious corals for curios and jewelry. Results of that assessment, along with a recommended strategy for action, were compiled in a report to the Task Force entitled, **International Trade in Coral and Coral Reef Species: The Role of the United States.** (The report is available at www.coralreef.gov)

While it is known that the US is the largest importer of coral reef fish for the marine aquarium trade, the magnitude of the trade is based upon estimates as almost all reef fish are not listed on CITES. The NMFS is analyzing the USFWS import data – recently modified to identify and separate out freshwater and marine fish – to assess the quantity of fish entering the U.S. The initial analysis indicates that more than twice as many coral reef fish are entering the U.S. for the marine aquarium hobby than was originally estimated, up to 16 million fish a year.

USCRTF Resolution 14-4 Call for Building Enforcement Capacity: At the 14th Meeting of the USCRTF in Palau, the Task Force called on its members to increase efforts to build enforcement capacity. The Steering Committee was charged with continuing training and other efforts, including the development of an enforcement “toolbox” in cooperation with the international Coral Reef Initiative (ICRI), to help coral reef management communities build enforcement capacity.

Statement Decision(s):

The USCRTF decides to:

- Form a working group on enforcement to assist in fulfilling Resolution 14-4.
- Identify and recommend specific experts in law enforcement, field forensics, and toxicology/biomarkers to serve on the working group from Task Force agencies.
- Utilize expertise from Task Force agencies to identify existing or potential cyanide detection methods or tests which could be used to determine if fish had been exposed to cyanide or other poisons.
- Explore the usefulness and need to convene a broader expert panel to resolve the issues associated with cyanide and other poison detection tests, and if needed, assist in convening such a panel.
- Encourage all coral reef jurisdictions to identify and promote alternative, sustainable, non-destructive practices in regard to aquarium fisheries.
- Experts and relevant representatives from Task Force Agencies will be identified and the Working Group on Enforcement formed within two months. Progress on assessing cyanide and other poison detection tests and building enforcement capacity will be presented at the next USCRTF Meeting.

