Centre for Environment Fisheries & Aquaculture Science

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World Class Science for the Marine and Freshwater Environment

Methods of detecting cyanide, thiocyanate and other by-products in seawater

A desk-based assessment

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Executive Summary

Cyanide fishing has been practised since the 1960s and the method is still thought to be used in at least 15 countries, including major live fish exporters such as Indonesia and the Philippines. Cyanide tablets are dissolved in bottles of seawater and squirted onto coral heads and into crevices to stun fish, thereby enabling their capture. Non-targeted species (e.g., corals and anemone) are also negatively impacted by cyanide exposure, making the use of this illegal fishing practice both an environmental and commercial concern which requires adequately evidenced management and enforcement.

In the late 1980s, the Philippines government and the International Marine life Alliance attempted to control this practice through the joint establishment of a set of Cyanide Detection Laboratories (CDL). However, the robustness of the methodology used for testing was challenged based on analytical problems including interferences and accuracy. In 2008, the National Oceanographic and Atmospheric Administration (NOAA) organized a Cyanide Detection Testing workshop to bring together experts and managers from key import and export countries to tackle the issues facing the regulation of cyanide fishing. Several recommendations were proposed, mainly in relation to the lack of knowledge of the cyanide toxicokinetics in marine fish and the lack of availability of a cheap, accurate and reliable method for the detection of cyanide exposure at relevant stages in the commercial chain.

Since the workshop a number of studies have worked towards increasing our knowledge base of cyanide toxicokinetics. However, most remain focused on the effects of human exposure with experiments being carried out using mammalian species, with very few targeting marine fish specifically. Gaps also remain in our knowledge regarding background levels of cyanide and metabolites in marine fish from regions at a high-risk to environmental cyanide inputs. Without this information it is very difficult to know when a fish has been exposed to cyanide and what levels of detection the method must be able to reliably achieve. Current effort is largely focused on the development of sufficiently sensitive and accurate sensors which are specific to the detection of cyanide in a variety of matrices.

There are, however, some promising studies that have developed methods for the analysis of cyanide and metabolites which could be reliably applied to marine samples. Some of these methods could be implemented using portable devices that would aid rapid results to be achieved in the field. However, further studies are required to improve our knowledge base, specifically in relation to cyanide toxicokinetics in marine fish. In addition, an improved understanding of the relationship between cyanide levels detected in the fish, in relation to background levels of cyanide naturally present in the environment from which the fish has

been extracted, is required to enable the likely source of any cyanide detected to be assessed and evaluated reliably.

Glossary

Adduct: the product of a direct addition of two or more distinct molecules, resulting in a single reaction product containing all atoms of all components.

Analyte: a substance that it is of interest for identification or measurement through an analytical procedure.

Anoxia: absence of oxygen.

Aromatic amine: organic compound consisting of an aromatic ring attached to an amine (e.g.: benzene, toluene, pyridine, etc).

Bioaccumulation: accumulation of substances which occurs when an organism absorbs a substance at a rate faster than that at which the substance is lost by metabolism and excretion.

Bioconcentration: the process by which a chemical concentration in an aquatic organism exceeds that in water as a result of exposure to a waterborne chemical.

Biomarker or biological marker: generally refers to a measurable indicator of some biological state, condition or process (such as metabolic process).

Biosensor: a device which uses biological materials (living organism or biological molecules, especially enzymes or antibodies) to detect the presence of chemicals. It converts a biological response into an electrical signal.

Chemosensor or molecular sensor: Is a molecule that interacts with an analyte to produce a detectable change.

Clean-up: a series of procedures a sample will undergo to eliminate as much matrix and interferences as possible, leaving a target compound as isolated as possible.

Corrin: a heterocyclic compound. It is the base of, for example, vitamin B_{12} (cobalamines).

Coumarin: a fragrant organic chemical compound from the benzopyrone family.

Cyclic voltammetry: an electrochemical detection technique (electrochemical) that measures the current in a cell.

Cryogenic trap or cold trap: a device that condenses vapors into a liquid or solid, preventing the loss of the vapor or getting the vapor in a more condensed/compact form.

Degradation: a change of a chemical compound into a less complex one, often breaking it down and deteriorating it.

Derivatization: a chemical process which transforms a chemical compound into a product with different properties, for example, a non-volatile into a volatile product.

Detoxification: the process of removing toxic substances.

Electrochemical detection: a technique that measures the electrons released after the oxidation or reduction of a compound.

Endogenous: having an internal cause or origin.

Exogenous: cause or origin is external.

Extraction: process of extracting something. In analytical experiments, extract a target compound from a sample or matrix.

Half-life: time required for any specified property (e.g., the concentration of a substance in the body) to decrease by half.

Headspace: a technique based on the collection (with a syringe or fibre) of the volatile compounds released from a liquid or a solid, usually on an enclosed space, like a sealed vial.

Hypoxia: diminished availability of oxygen to the body tissues.

Indole: aromatic heterocyclic compound consisting on a benzene fused to a pyrrole ring with a nitrogen. E.g., the tryptophan, a precursor of the neurotransmitter serotonin.

Interference: a substance that can be measured at the same time than a target compound but usually prevents or difficulties the measurement of the target compound.

Internal standard: a compound added to both samples and standards at a known concentration in order to provide a basis for comparison when doing the quantification of the samples.

Interspecific: characteristic occurring or existing between different species.

Intraspecific: characteristic occurring or existing within a species or individuals from a single species.

Isotopic analysis: identification of the distribution of certain stable isotopes and chemical elements within chemical compounds.

Limit of detection: the lowest concentration a technique or detection system can measure a target compound. The signal (peak) must clearly be due to a compound/analyte and not background noise coming from the detector.

Linear range: range of concentrations where the relationship between the concentration and the signal produced is linear (x= a + by).

Matrix: components of a sample other than the analyte of interest.

Mass spectrometry: a detection technique based on the measurement of the masses of molecules and molecular fragments after being ionized.

Metabolite: any substance, intermediate and product, involved in metabolism.

Metabolization: chemical processes occurring within a living cell or organism that are necessary for the maintenance of life. During metabolism, some substances are broken down to yield energy, some are synthesized and some are transformed into less toxic substances.

Pharmacokinetics: a branch of pharmacology concerned with the movement of drugs within the body.

Photolysis: decomposition or separation of molecules by the action of light.

Porphyrin: a group of heterocyclic pigments containing four pyrrole rings, sometimes with a central metal atom. E.g.: heamoglovgin and chlorophyll.

Pretreatment: a series of steps or treatments a sample will receive to be able to analyse the target compounds.

Polymeric membrane: a semi-permeable membrane that allows the separation of certain compounds from a solution depending on its charge and porosity.

Recoveries: amount (usually as a percentage) of target compound not lost during a procedure. E.g., how much compound is measured in a sample with an known added amount of target compound after extraction and clean-up.

Sample: representative fraction or part of a larger whole or group subject of a study. E.g., a fragment of muscle from a fish or an aliquot of a bottle of liquid.

Selectivity: ability of a technique to detect or measure a target compound without interferences from other compounds.

Sensitivity: measurement of the capability of a technique to see and/or measure a target compound at very low levels. It is often expressed by the limit of detection.

Separation technique: technique that allows the separation of different compounds in a sample based on different properties, such as charge, size, etc. It reduces the amount of interferences reaching a detector. E.g., gas and liquid chromatography.

Solid phase extraction: a method of extraction that uses a solid material and a liquid to isolate a compound from a solution. It is usually used to extract a compound from a large amount of liquid or to clean a liquid sample isolating the compound from the rest of the sample.

Spectrometry: a method used for the study of certain arrays of entities (spectrums), as light waves or particles. Ion-mobility spectrometry, mass spectrometry, Rutherford backscattering spectrometry and neutron triple axis spectrometry are the main forms of spectrometry.

Spectrophotometry: a quantitative measurement of reflection and transmission properties of materials as function of the wavelength.

Spectroscopy: the study of the interaction between matter and radiated energy, in other words, the study of systems by the electromagnetic radiation with which they interact or that they produce.

Spirooxanzine: a group of molecules which modify their structure upon exposure to light changing colour reversibly (photochromism).

Standards: materials contain a precise amount (concentration) of a compound of use in quantitative analysis. Standards provide a reference that can be used to determine unknown concentrations or to calibrate analytical instruments.

Taurine: an amino acid containing sulphur and important in the metabolism of fats.

Toxicokinetics: description of the processes a chemical undergoes once inside the body. More specifically, the rate a chemical will enter the body and the processes and rate the chemical is metabolized and excreted.

Volatile: a substance that is readily vaporizable at relatively low temperature.

1. Detection of cyanide in seawater: why are we interested?

Cyanogenic compounds are present in the marine environment as a result of both natural and anthropogenically derived pathways. Naturally, they are produced by bacteria, fungi, algae, vascular plants and some invertebrates (Kamyshny et al., 2013), although in small quantities which are unlikely to attain toxic concentrations in in the aquatic environment (Knowles 1976; Dzombak et al., 2006). Cyanogenic glycoside compounds have also been documented in over 2,500 species of plant, across different taxonomic groups, including cassava, apricots, cherries, apples and sweet potatoes (Vetter, 2000) with recorded cyanide concentrations of 2,360,000 and 3,000,000 µg/L in cassava and lima beans respectively (WHO, 2004). The natural breakdown of cyanogenic glycosides within plants is generally considered to occur in quantities insufficient to cause high levels of cyanide in the surrounding environment. However, a study by Kamyshny et al. (2013) recorded concentrations of both free and complexed cyanide up to 6.94 µmol/L (1.92 µmol/L or 82.6 µg/L free cyanide) in saltmarsh sediments due to the decomposition of the cord grass, *Spartina alterniflora*. The roots were found to contain 50 µmol/kg cyanide at the time of sampling. It is possible that other similar aquatic habitats, such as mangroves forests, are also capable of accumulating cyanide compounds in sediments. However, no evidence currently exsists to support this.

The burning of biomass, such as during forest fires, is perhaps the greatest natural input of hydrogen cyanide into the atmosphere. High temperatures attained during burning events causes pyrolysis which liberates hydrogen cyanide from amino acids, nitrogen heterocycles, dicarboxylic acids and cyanogenic glycosides (Johnson and Kang, 1971; Barber et al., 2003). Biomass burning releases an estimated 1.4 – 2.9 Tg N/yr, and after an atmospheric lifetime of around five months, the ocean is reported to be the primary sink capable of absorbing 1.1 – 2.6 Tg N/yr (Li et al., 2000; Li et al., 2003). Oceanic concentrations of atmospherically derived cyanide, whilst not fully understood, are predicted to be less than 1 µg/L (Li et al., 2000; Dzombak et al., 2006). Whilst much of the cyanide from biomass burning is released into the atmosphere, both gaseous and particulate cyanide contained within ash deposits may also diffuse or be washed into aquatic environments through wet and dry deposition and precipitation run off (Barber et al., 2003).

Cyanide can also enter the marine environment as a result of anthropogenic activities due to its use in a wide range of industrial processes, such as in iron and steel processing plants, metal electroplating, the extraction of gold and silver from ores, petroleum refineries, the

manufacture of synthetic fibres such as nylon, plastics, fertilisers and pesticides, as well as in an illegal method of fishing (i.e., cyanide fishing) (Eisler, 1991). From an environmental management perspective and in order to effectively regulate it's illegal use in fishing practices, it is important to understand the naturally occurring background levels of cyanide, particularly in areas where anthropogenically derived cyanide is present in elevated concentrations. In the following section, the major sources of anthropogenically derived cyanide are discussed.

1.1. Anthropogenic sources of cyanide and associated by-products

1.1.1. Industrial processes

Cassava, the third most important source of calories in the tropics after rice and maize (FAO, accessed 11/07/2017), is able to grow in poor soil conditions throughout much of the tropics. During cassava production, cyanogenic glycosides are released and degrade into hydrogen cyanide. The process can result in waste water concentrations of cyanide up to 200,000 µg/L (Simeonova and Fishbein, 2004; Siller and Winter, 1998) and cyanide in concentrations of up to 7,890 µg/L have been detected in waters adjacent to cassava processing plants in Nigeria (Okafor et al., 2001). Therefore, waste water effluent from cassava processing plants has the potential to be an important, but localised, source of hydrogen cyanide entering the aquatic environment.

Metal processing and mining activities are considered to be the greatest industrial source of cyanide entering the natural environment (Dash et al., 2009). In mining, cyanide has been used to extract precious metals from their ores for over a hundred years and is particularly prevalent in gold and silver mining industries with over 90 percent of gold excavated globally extracted using cyanide (Mudder and Botz, 2004). When cyanide is used to extract gold from ores it will typically bind with other common metals also present to produce a huge range of different cyanometal complexes. These complexes vary in strength, weak complexes (weak acid dissociable) typically breakdown in acidic conditions, while stronger complexes, such as those formed with iron and gold are much more stable and are degraded by exposure to light at particular wavelengths (Moran, 1999; Moran, 2001). Cyanometal complexes are much less toxic than either the free cyanide ion or hydrogen cyanide. However as they break down they release free cyanide.

Thiocyanate, the major product of cyanide metabolism in aerobic organisms, can also be produced as a result of mining activities. However, the formation of cyanometal complexes interferes with this pathway and may inhibit the formation of thiocyanate (Johnson, 2015).

Cyanide can also react with sulphides which are sometimes present in mining materials to form thiocyanate. While less toxic than free cyanide, thiocyanate has been shown to be toxic to various aquatic organisms, particularly fish (Rubec, 1986; Lanno & Dixon, 1996). It is also more stable than free cyanide so it persists in the environment for longer, potentially dispersing over a wider area (Gould et al., 2012). Large scale pollution events can also be associated with mining activities and can introduce huge quantities of cyanide compounds into the environment over a short time period. These events are associated with large scale fish kills which have historically been observed globally.

Cyanide pollution resulting from industrial processes is most commonly monitored through the collection and analysis of water samples. In 1983, a study in Jiaozhou Bay, China was conducted to determine cyanide concentrations in the bay. The two main cyanide inputs were reported to be riverine as well as from industrial activities within the bay area itself. Low, but detectable, cyanide concentrations within the bay of 0.02 - 0.46 µg/L were recorded with lower concentrations in the mouth of the bay as distance from the assumed cyanide source increased (Yang et al., 2015; Yang et al., 2016). Rivers are the major pathway for the transfer of cyanide from inland mining activities to the marine environment. A study by Lu in 2014 found that 92 percent of water samples collected from different freshwater sources in the Benguet mining district of the Philippines (N=98) contained detectable concentrations of cyanide. However, none of these samples exceeded the maximum allowable limit for free cyanide (200 µg/L). Recent work carried out by Mansfeldt and Hohener (2016) aimed to identify different sources of cyanide (commercial, blast furnace, coal-carbonization) using isotopic analysis. However, while they could differentiate commercial from non-commercial cyanide, they could not differentiate between the various different commercial sources.

1.1.2. Fishing

The use cyanide in the form of sodium cyanide (NaCN) or potassium cyanide (KCN) to collect live fish was first documented in the Philippines in the early 1960s for the capture of live fish for the marine aquarium trade (Rubec, 1986; Halim, 2002). By the 1970's, the technique had spread throughout Southeast Asia and into the Pacific islands for use in the collection of live reef fish, not only to supply the aquarium trade but also for the live reef fish food trade (LRFFT) which targets large groupers, coral trout and humphead wrasse harvested for human consumption (Bruckner & Roberts, 2008). Estimates suggest that by the mid-1980s, more than 80 percent of all fish harvested in the Philippines to supply the aquarium trade were collected using cyanide and by the mid-1990s, approximately 90 percent of vessels transporting live fish in the eastern islands of Indonesia had cyanide on board (Wabnitz et al., 2003). Today, it is believed that cyanide fishing is still used by some to

collect live fish despite laws in most exporting countries prohibiting its use (Ochavillo et al., 2004; Calado et al., 2014; *pers.comm* Rose-Liza Eisma-Osorio, Professor of Environmental Law, University of Cebu, Philippines July 2017).

The technique used by some aquarium trade collectors is achieved by mixing one or two crushed cyanide pellets with seawater in “squirt” bottles, which results in a hydrogen cyanide (HCN) solution. This solution acts on the fish by interfering with oxygen metabolism, blocking the key enzyme system cytochrome oxidase (Mak et al., 2005), and is released into cracks and crevices within the reef framework by divers to target and stun desirable fish which would otherwise be difficult to collect. Once at the surface, the fish are transferred to clean, cyanide-free water for recovery on board the fishing vessel (Watnitz et al 2003).

The technique used for the LRFFT differs somewhat and is described in Calado et al (2014) as being conducted on a larger-scale and having a greater impact to the surrounding non-target environment. Fishermen fill 100–200 L plastic/metal drums with a cyanide solution and load them onto small fishing boats. On arrival at the fishing site, divers set up gill or barrier nets around the coral reef before the boat circles the reef releasing the cyanide solution, thereby stunning fish in the area and allowing them to be easily collected by hand-netting from boats or by divers.

Cyanide is toxic to fish and although their ability to detoxify the substance enables them to ingest high, sub-lethal doses (Eisler, 1991 in Wabnitz et al., 2003), exposure to the solution can have irreversible effects on fish condition which may result in subsequent death (Way et al., 1988 in Wabnitz et al., 2003). Wabnitz et al., (2003) reported that the number of fish collected using cyanide which die within hours of collection can range from between 5 to 75 percent, with on average another 30 percent dying prior to export. However, acquiring recent, robust data on cyanide-related mortality of traded reef fish is particularly difficult given the Illegal, Unreported and Unregulated (IUU) nature of the method.

In addition to the direct and relatively short-term effects of cyanide on the target fish there are a number of other environmental concerns associated with its use. The population status and viability of moderately abundant target fish species collected for the aquarium and live fish for food trade using cyanide, such as the humphead wrasse *Cheilinus undulates,* have been severely reduced with increasingly prevalence of cyanide fishing in South-East Asia (Hodgson, 1999). Non-targeted species, including corals and other invertebrates, are also negatively impacted by cyanide use. Much lower cyanide concentrations than those used for fishing have reportedly caused mortality to corals and anemones and brief exposures to cyanide can produce long-term damage (Cervino et al. 2003). Corals exposed to cyanide have been found to have a reduction or cessation of respiration with the most obvious

response being the dissociation of the coral-algal symbiosis resulting in discolouration or bleaching. The ecological consequences of this is observed to include a reduction in phototrophic potential, a decrease in growth rates and a decrease in fecundity with recovery of symbiosis taking between six months to one year or more (Jones 2006). Cyanide fishing also poses a risk to human health as the fishermen deploying the solution are regularly diving in close proximity to the poison and the technique is often associated with compression diving (oxygen pumped from the surface through a hosepipe to the diver below) (Halim, 2002; *pers.comm* Rose- Liza Eisma-Osorio, Professor of Environmental Law, July 2017).

KEY POINTS

- Cyanide is present in the marine environment as a result of both natural (e.g. produced by plants or an output from forest fires) and anthropogenically derived pathways (waste waters from industry or from cyanide fishing).
- Cyanide fishing for live fish collection was first documented in the Philippines in the early 1960s where some fishers mix crushed cyanide pellets with seawater in “squirt” bottles resulting in a hydrogen cyanide (HCN) solution which stuns fish making them easier to catch.
- The solution acts on the fish by interfering with oxygen metabolism, blocking the key enzyme system cytochrome oxidase.
- Non-targeted species like the corals and other invertebrates are also negatively impacted by cyanide use.

1.1.3. Overview: Detecting the use of cyanide to collect live fish

Due to the known significant impacts of cyanide fishing on target species, associated reef habitats and species, and human health, the technique is banned in most exporting countries with 77 percent of 18 key exporters having anti-cyanide laws (Dee et al., 2014). Efforts to mitigate the use of cyanide in the Philippines (one of the top exporting countries) began in the 1990s, when the government, in collaboration with the International Marine life Alliance, implemented a permitting system and cyanide testing initiative. They established a number of Cyanide Detection Laboratories (CDL) to test reef fish at export (Barber and Pratt, 1997; Barber, 1999; Rubec et al., 2001). However, as the relaible detection of cyanide is so difficult, the accuracy and robustness of the testing units was questioned (Mak et al., 2005; Bruckner and Roberts, 2008) and currently only four cyanide testing labs are functional in the country and are used for other purposes (*pers.comm* Rose-Liza Eisma-Osorio, Professor

of Environmental Law, University of Cebu, Philippines July 2017). In addition, complex supply chains, the problematic nature of detecting cyanide with increasing time post collection and knowledge gaps in environmental background levels of cyanide through natural and industrial processes, present priority challenges for law enforcement when gathering robust evidence of cyanide fishing activity (Dee et al., 2014). However, the ornamental industry (for the hobby and public aquaria) remain committed to ensuring that their suppliers are providing sustainable and legally collected fish which are harvested in line with currently accepted best practise.

1.1.4. Cyanide Detection Testing Workshop, 2008

In response to the knowledge gaps in the robust detection of cyanide in reef fish resulting from fishing, a Cyanide Detection Testing Workshop was conducted in 2008 in the USA, the world's largest importer of marine aquarium fish. The International workshop, funded by the National Oceanographic and Atmospheric Administration (NOAA) Coral Reef Conservation Program and Kingfisher, was held in Florida and brought together a range of cyanide technical specialists including forensic chemists, government and non-government representatives from the USA and three of the biggest exporting countries, the Philippines, Indonesia and Vietnam. The workshop aimed to mitigate the use of cyanide to capture reef fish for both the marine aquarium trade and the live reef food fish trade and provided nine priority recommendations for working towards a robust method for detecting cyanide fishing activities. A summary of the recommendations from the workshop is provided in Table 1.

Table 1. Summary recommendations from the Proceedings of the International Cyanide Detection Testing Workshop in the USA in 2008 (Bruckner & Roberts, 2008).

	Recommendation	Key challenges
1	Determine the pharmaco-kinetics of cyanide	Determine detectable level of cyanide and metabolites in coral reef fish and how levels change over time following exposure. Determine detectable environmental background levels. Establish the effect of cyanide detection following freezing of samples
2	Validation of the Ion Selective Electrode (ISE) cyanide detection test	Establish the sensitivity, specificity, accuracy and precision of the ISE method which was commonly used in the Philippines between 1993 to 2001.
3	Establish networks of Cyanide Detection Testing (CDT) laboratories at points of export	Develop standardised testing at export points (airports).

	Recommendation	Key challenges
		Include poison-free capture testing in export licenses to ensure compliance and self finance the cost of testing.
4	Obtain funding for establishment and maintenance of CDT networks in exporting countries	Obtain funding from, for example, World Bank, Asian Development Bank or international NGOs.
5	Establish training, quality assurance and proficiency programs for CDT labs and chemists	Development of a training program. Development of an ISO accredited quality assurance program.
6	Explore options for a simple field test at points of collection	Develop a field test to detect presence of cyanide (not concentration) at collection points and holding facilities.
7	Testing and accreditation plan for cyanide free fish in exporting countries	Establish a certification scheme funded by suitable export license fees. Develop a cyanide testing database and link license renewal to the testing program.
8	Implement complementary legislation against cyanide fishing in importing countries	
9	Develop a cyanide testing program in the United States	

These recommendations focused on improving knowledge relating to toxicokinetics of cyanide and its metabolites, as well as the need for new analytical methods which could be used at both export and import hubs to accurately determine when fish have been exposed to cyanide. There was particular interest in establishing a simple field test which could be used at collection points before the illegally caught fish entered the supply chain.

In addition to the recommendations presented above, Logue et al. (2010) identified several factors to consider when selecting a biomarker and an analytical method for the analysis of cyanide exposure: metabolism (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.

For the purpose of this review, we will focus on three of these points:

- The target compound(s) to monitor and analyse. This may depend on where the method will be used (laboratory or field, in the export or the import country);
- The level at which the target compound(s) is/are expected to be which will determine which technique or method can be used depending on how sensitive it is; and

- The matrix in which to monitor the target compound(s). This will influence the levels at which the target compound is found and would possibly interfere with the detection method. The best option would be a non-invasive and non-destructive method.

1.1.5. Project aim and objectives

The aim of this desk-based study is to review analytical methods which are currently available for detecting cyanide and its associated by-products, namely in aqueous matrices such as seawater. Specifically, the report will:

1. Identify articles for review using Science Direct, Web of Science, Google Scholar and other sources focussing on ISI listed journals according to defined criteria;
2. Assess each technique considered and assign a “quality” value according to a modified Klimisch score; A Systematic Approach for Evaluating the Quality of Experimental Toxicological and Ecotoxicological Data (Klimisch et al., 1997). Categories include; 1: Reliable without restriction, 2: Reliable with restriction, 3: Not reliable, 4: not assignable;
3. Provide recommendations based on the assessment regarding which method/s may be suitable for further development and what that would involve.

2. Cyanide: the target compound and associated by-products

2.1. Cyanide (CN^-) **C≡N**

Cyanide is any chemical compound that contains CN. Organic forms of cyanide are typically referred to as nitriles, so the term cyanide is used mainly for the inorganic forms. Inorganic cyanides, such as sodium or potassium cyanide, are negatively charged ions (CN^-). Inorganic forms of cyanide release CN^- and are very toxic whereas organic cyanides do not release CN^- and are less toxic or, in the case of insoluble polymers, nontoxic. The most hazardous compound is hydrogen cyanide (HCN) which is a gas at ambient pressure and temperature and can be produced when a solution containing a labile cyanide is made acidic.

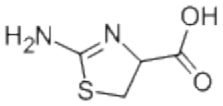
Cyanide is reported to be the only biomarker capable of indicating exposure of cyanide within the initial minutes following exposure in humans and animals (Logue et al., 2010). However, it is rapidly metabolised to thiocyanate (SCN^-) and ATCA, so is difficult to detect once significant time has elapsed following exposure (Logue et al, 2010). Cyanide is distributed via the blood stream and concentrates in tissues and organs with the highest blood flow, such as the liver, kidney, brain and muscles, where it binds to the enzyme rhodonase, producing thiocyanate (SCN^-) (Bruckner and Roberts, 2008). Blood and tissues have been the preferred matrices for cyanide analysis and detection. However, it is difficult to assess rates of cyanide decay in tissues because levels of rhodonase are variable between organs, decay with time, and can also be bound to tissues (Logue et al, 2010). Another challenge associated with the detection of cyanide results from the fact that levels in the sample can be altered up to 66 percent during 14 days of storage and can be artificially formed during storage from thiocyanate (Logue et al., 2010). Therefore, cyanide should be analysed as soon as possible after collection and preserving techniques must be considered to provide accurate concentrations.

2.2. Thiocyanate (SCN^-) **S═C═N**

Thiocyanate is analogous to the cyanate ion $[OCN]^-$, where oxygen is replaced by sulfur $[SCN]^-$. Thiocyanate is produced by the reaction of elemental sulfur, cystine or thiosulfate with cyanide. But thiocyanate can also be formed from cyanide in enzymatic process by the sulfur transferase system. Reversely, it can be oxidized at acid pH by hydrogen peroxide generating sulfate and cyanide. Thiocyanate is the main detoxification product of cyanide in organisms, with longer half-lives than cyanide and appreciable concentrations that can be

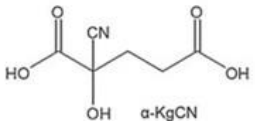
found in biological matrices “shortly” after exposure (Logue et al., 2010). Most studies found a progressive increase in SCN^- in plasma over multiple days, followed by a decline until a period where it was no longer detectable (from 16 days to 16 weeks or more) (Bruckner and Roberts, 2008). One of the main issues in usingthiocyanate concentrations as a proxy for cyanide detection is that it is naturally found in biological fluids, and although this is true for all cyanide metabolites, thiocyanate levels are relatively high and can be inconsistent. The variability of background levels also makes it difficult to determine low-level cyanide exposure (Logue et al., 2010). Also, due to the SCN^- half-life and rapid production from cyanide, thiocyanate has been found to bioaccumulate in living organisms (Brown et al., 1995; Lanno and Dixon, 1996). Studies of thiocyanate in river fish have shown that these fishes are capable of accumulating thiocyanate against its concentration gradient (bioconcentrate) and at relatively rapid rates depending upon the exposure SCN^- concentration, fish size and anionic composition of the exposure water (Heming et al., 1985). In addition, thiocyanate can be interconverted into cyanide and cyanide can be converted into thiocyanate during storage, although some precautions can minimize these interconversions (Logue et al., 2010). Most SCN^- is excreted in urine (Bruckner and Roberts, 2008), so it could be found in the fish tank water. Toxicity of thiocyanate is associated to its transformation into cyanide but thiocyanate will affect the thyroid gland decreasing the amount of thyroxine produced. At the same time, reduced or complete absence of thiocyanate can damage the human defence system.

2.3. ATCA (2-amino-2-thiazoline-4-carboxylic acid)



The second most abundant metabolization product from cyanide, which can be used as an alternative to SCN^- for determination of cyanide exposure, is ATCA. This compound has been hypothesised to be produced when there is a limited abundance of the enzyme rhodonase and/or limited abundance or access to sulphur which is necessary to produce SCN^-. It is not reported to be involved in other biological processes so it is not metabolized further. It is stable in stored biological samples for months of freezing and at ambient temperatures so it could provide a lasting signature of cyanide exposure (Logue et al. 2010). However, relatively few techniques have been described to analyse ATCA from biological matrices and relatively few studies have been conducted to evaluate the relationship between ATCA and cyanide exposure (Logue and Hinkens 2008). The concentrations of ATCA in different mammals (rat, rabbit and pig) following cyanide exposure have been found to be variable, with up to a 40-fold increase observed in rabbits, while ATCA in rats did not significantly rise above baseline levels (Bhandari et al., 2014). Its importance and production

is therefore very species-dependent. To date, it has not been used as a biomarker for cyanide exposure due to little scientific understanding of its toxicokinetics (Logue et. al, 2010) but its concentrations are very low and will therefore require a very sensitive method (Jackson and Logue, 2017). It has been suggested that ATCA is neurotoxic and could contribute to cyanide toxicity.



2.4. Other biological by-products and activities

Although thiocyanate and ATCA are the main metabolites produced after exposure to cyanide in mammals and freshwater fish, studies have found the presence of other compounds that are less important in terms of abundance, but that could be very specific for the exposure to cyanide based on studies in mammals. If these were stable, they could serve as long-lived markers of cyanide exposure (Logue et al., 2010). However, their use is currently very limited because there is no information about their production or importance in marine fish. They are also naturally produced in biological fluids and some are not excreted (in urine, for example), which means they require the analysis in tissues.

α-ketoglutarate cyanohydrin (α-KgCN): Concentrations in mammals are quite low and variable compared to cyanide and SCN-. Its low concentrations will require a very sensitive method (Jackson and Logue, 2017).

Cyanide-protein adducts: The minor metabolic endpoints for cyanide, so analysis will require a very sensitive method (Jackson and Logue, 2017).

Cytochrome oxidase: Dose-related reductions in cytochrome-c-oxidase activity were detected in various organs of rats exposed to oral doses of potassium cyanide. This marker was suggested as a method of diagnosis for samples taken within two days post-mortem (Debenham 2008).

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. (Debenham 2008).

Vitamin B-12 (Jackson and Logue, 2017): Another metabolite produced at the same time as ATCA is 2-iminothiazolidine-4-carboxylic acid (ITCA), the tautomeric form of ATCA. Formate and gill protein (Debenham 2008) have also been described as potential biomarkers after exposure to cyanide.

KEY POINTS:

There are several potential target compound indicators or biomarkers of cyanide exposure. However, all have pros and cons:

- cyanide can be degraded or metabolized very quickly, so it is not suitable for analysis when several days have been passed since exposure;
- thiocyanate is the metabolite most abundantly produced, but samples will have levels non-related to cyanide exposure due to natural production in the organism or interconversion from cyanide.
- Other metabolites could be more realiable indicators of exposure to cyanide, but they are also naturally produced in biological fluids and there is currently insufficient information about their production or their importance specifically in marine fish.

2.5. Chemistry and metabolism of cyanide

The cyanide solution most commonly used by illegal fishers for the capture of live fish is in the form of sodium cyanide (NaCN) (Philippines) or potassium cyanide (KCN) (Indonesia). A similar form of NaCN is also used in mining sites. This white solid dissolves readily in water, yielding sodium ion (Na^+), and cyanide ion (CN^-). Some of the CN^- then converts into hydrogen cyanide (HCN). The cyanide ion (CN^-) and hydrogen cyanide (HCN), are often collectively called free cyanide, and the relative amounts present are largely controlled by the water pH: CN^- is the predominant stable form of free cyanide above pH 9.0-9.5, starting to transform into HCN as the pH drops to be about 99.5 percent of the free cyanide at pH of 7.0 (Moran & Robert, 2000). Since the natural pH range in coral reef waters is between 7.0 and 8.5, most free cyanide in natural waters is present as HCN, some of which is released into the air (Figure 1). Cyanide readily combines with most major and trace metals. Some of them, including the iron-cyanide, can be decomposed by photolysis and release free cyanide. Because of the cyanide anion's high nucleophilicity, cyano groups are readily introduced into organic molecules by displacement of a halide group, forming groups called nitriles, such as N≡C-Cl or N≡C-O-R. Cyanide has also breakdown compounds, including free cyanides, metal-cyanide complexes, cyanogen chloride, cyanates, chloramines, ammonia and, most importantly in our case, organic-cyanide compounds and thiocyanates (Moran & Robert, 2000).

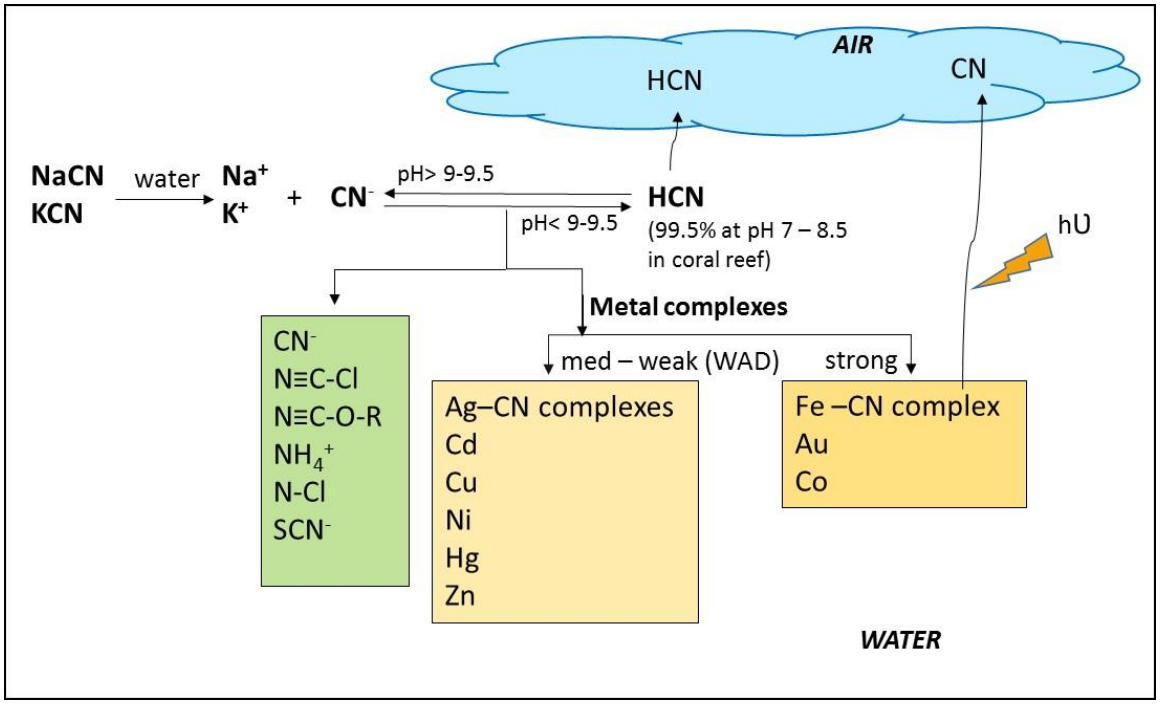


Figure 1. Cyanide routes and reactions in the aquatic environment. NaCN (Sodium cyanide); KCN (Potassium cyanide); Na+ (Sodium ion); K+ (Potassium ion) CN- (Cyanide ion); HCN (Hydrogen cyanide); N≡C-Cl or N≡C-O-R (Nitriles); NH4+ (ammonia); N-Cl (chloramines); SCN- (Thiocyanate); Ag (Silver); Cd (Cadmuim); Ni (Nickel); Hg (Mercury); Zn (Zinc); Fe (Iron); Au (Gold); Co (Cobalt).

Once cyanide is absorbed by the fish it acts by interfering with oxygen metabolism by blocking the cytochrome oxidase, causing cytotoxic tissue hypoxia and anoxia. Detoxification quickly begins, helped by the enzyme rhodonase, with a reaction between cyanide and rhodanese to produce the metabolite thiocyanate (SCN^-) which is excreted in the urine. Most studies relating to metabolization and half-lives of cyanide and related compounds are currently reported in mammals or fresh water fish (Figure 2). Studies in mammals have shown that cyanide is metabolized in a matter of hours to SCN^-, and nearly 80 percent of all cyanide entering the organism is converted to SCN^- which is then excreted in the urine (Logue et al., 2010). Other minor metabolites include the 2-amino-2-thiazoline-4-carboxylic acid (ATCA) and the reversible reaction of cyanide with hydroxocobalamin to form cyanocobalamin (a form of vitamin B_{12}) (Logue et al., 2010).

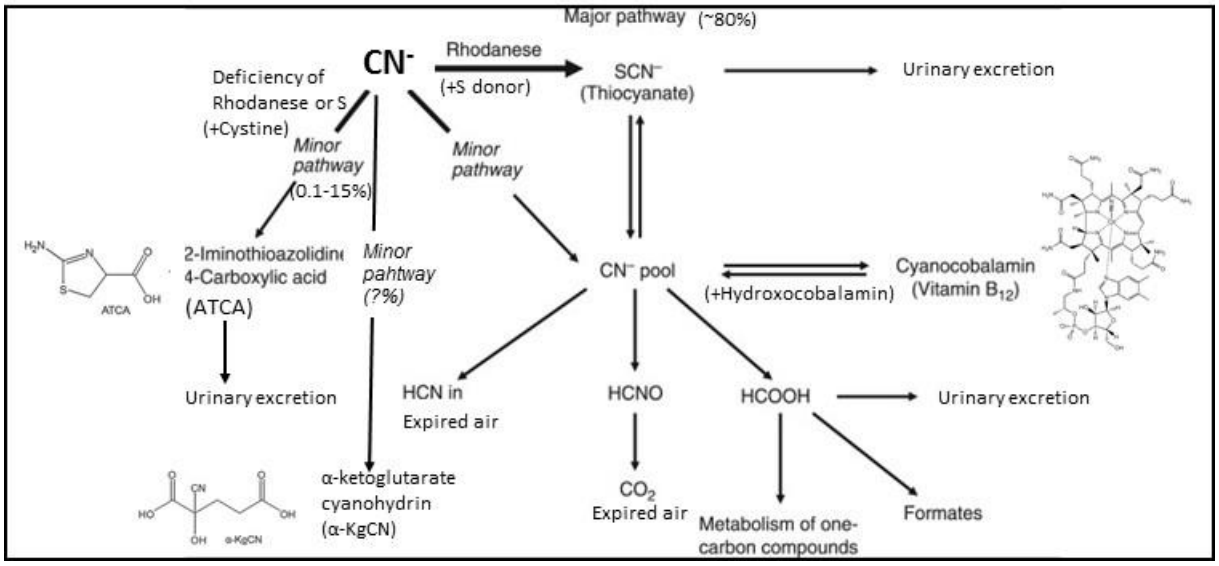


Figure 2. Pathways of the cyanide metabolism and elimination in mammals and freshwater fish (Modified and adapted from Isom et al. 2010 and Logue et al. 2010).

Although the metabolism and by-products of the cyanide are known, the toxicokinetics of the cyanide, i.e. the rate different metabolites are being produced and their importance, are not well understood. Some studies have attempted to fill this gap for example, Mitchell et al. (2013a) conducted experiments with swine studying the levels of cyanide, SCN^-, ATCA and α-ketoglutarate cyanohydrin (α-KgCN) in plasma after exposure to cyanide. They saw a rapid increase of α-KgCN during infusion of cyanide, indicating that it is quickly converted to α-KgCN. However, when the cyanide infusion stopped, the concentrations of α-KgCN decreased rapidly like ATCA. SCN^- concentrations also decreased after exposure until the conversion of cyanide into SCN^- was quicker than the excretion of SCN^- (Figure 3).

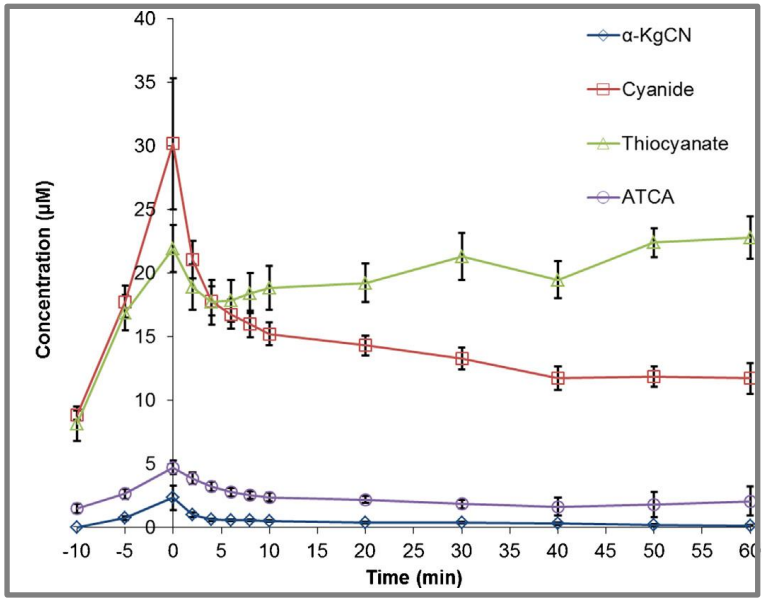


Figure 3. Toxicokinetic profile of α-KgCN, cyanide, thiocyanate, and ATCA in control swine. Apnea, pre-exposure and 5 min infusion sample points are designated as "time 0, −10, and −5", respectively. The plasma sampled at time zero was drawn prior to treatment, the −10 time point was obtained before infusion and the −5 time point was collected 5 min after exposure (Mitchell et al., 2013).

Bhandari et al. (2014) carried out cyanide exposure experiments with rats, rabbits and swine studying the levels of cyanide, SCN^- and ATCA. Figure 4. Rat whole blood cyanide, thiocyanate and plasma ATCA normalized concentrations after cyanide exposure (6 mg/kg body weight KCN injection subcutaneously to rats). Inset: Full time course up to 50.5 h post-injection of KCN. and Figure 5 show the evolution of the levels of cyanide, thiocyanate and ATCA in rat, rabbit and swine blood. These figures highlight how different the profiles were, reinforcing the variability in the toxicokinetics between different species. All animals showed a maximum of cyanide in the blood followed by a rapid decline. Thiocyanate concentrations increased more slowly as cyanide was enzymatically converted to SCN^-. ATCA concentrations varied significantly between animals, with no significant increase in rats but increases of 40 and 3-fold in rabbits and swine respectively. ATCA concentrations then fell rapidly similar to that of the concentrations of cyanide. Thiocyanate seems to be the only compound that consistently had increased levels after exposure to cyanide, although again the results for all animals and compounds were species dependent.

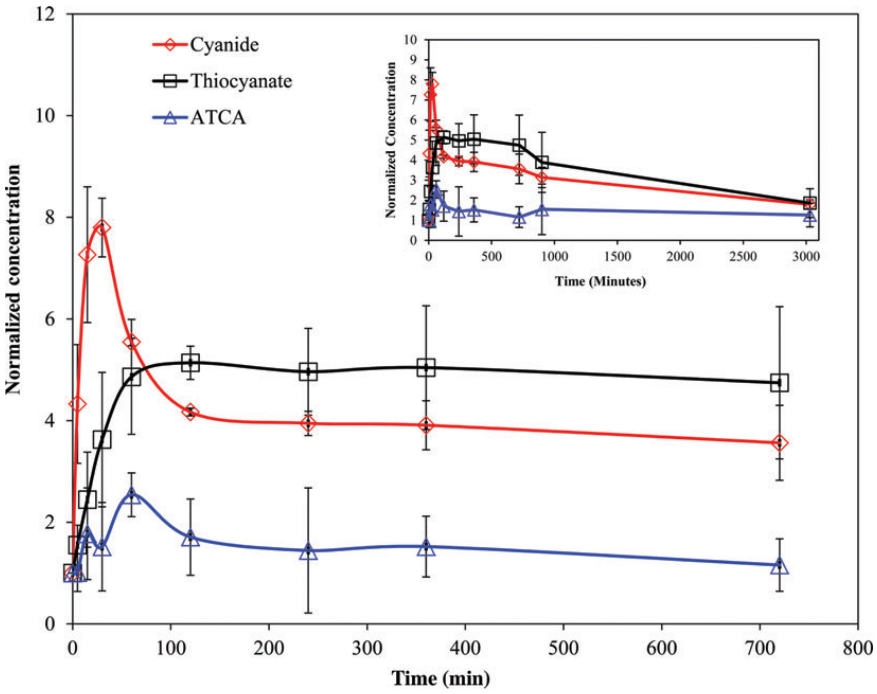


Figure 4. Rat whole blood cyanide, thiocyanate and plasma ATCA normalized concentrations after cyanide exposure (6 mg/kg body weight KCN injection subcutaneously to rats). Inset: Full time course up to 50.5 h post-injection of KCN.

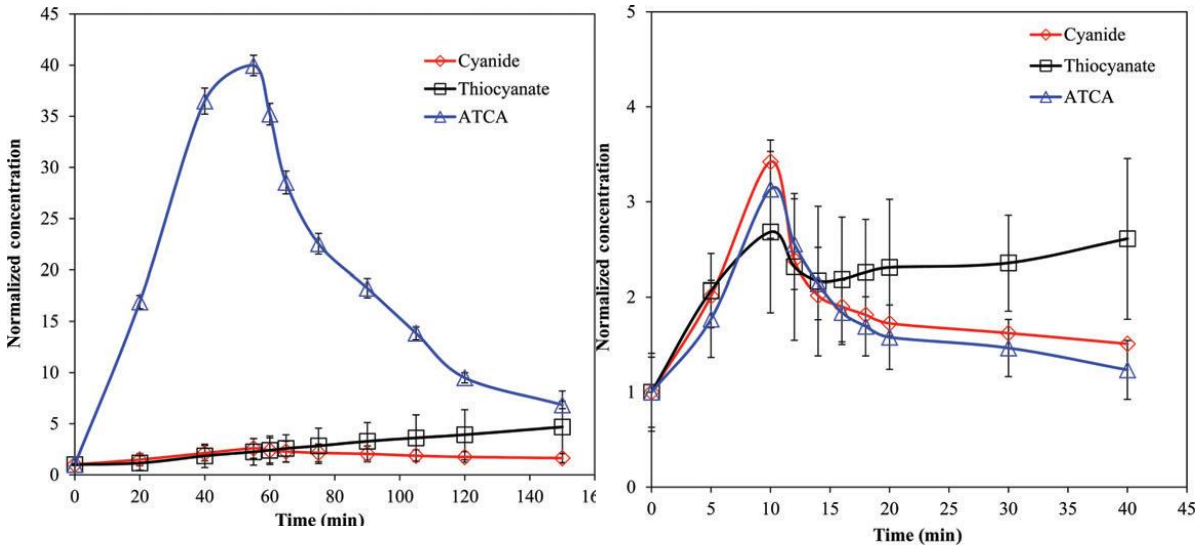


Figure 5a (Left). Plasma cyanide, thiocyanate and ATCA nor- malized concentrations after 10 mg NaCN infusion to rabbits (Bhandari et al., 2014). Figure 5b (Right). Swine plasma cyanide, thiocyanate and ATCA normalized concentrations during and after intravenous dose (0.17 mg/kg/min until apnea; ~10 min) (Bhandari et al., 2014).

Since most studies have focused on mammals or fresh water fish, there is still controversy about the half-lives of some of these compounds in marine fish. If results from metabolism in mammals were extrapolated, cyanide would metabolize within a matter of hours in the organism following exposure. ATCA would be an important metabolite, but in much lower concentrations than thiocyanate which remains the major metabolite. However, extrapolation must be considered with caution as there are differences in the effects, products and rates of metabolization between different mammal species, so it is very likely that the results cannot

reliably be applied to marine fish. In addition, although cyanide has been reported to disappear in a matter of hours in mammals, it has been found to be present in whole fish samples up to 5-14 days after exposure during routine analysis (Rubec et al., 2003). Targeted studies of metabolism by marine fish, and ideally species-specific experiments, are still necessary to determine the most robust target compounds and also to establish half-lives of metabolites to determine when they can be reliably detected in samples at varying time scales following initial exposure.

KEY POINTS:

- Once it enters aquatic media cyanide has a very short life: it is very reactive, so it will form metal complexes, organic derivatives (such as SCN-) or it will enter biota.
- In the organism, cyanide is quickly metabolized producing compounds such as SCN- , ATCA or α-KgCN. Most studies have focused on mammals and freshwater fish and include information on; how these metabolites are produced, how long they last, what their production rate is and their importance in relation to the exposure to cyanide.
- In mammals, SCN- is the only compound with consistenly increasing concentrations after exposure while ATCA production was very variable.
- Differences between mammalian species means extrapolating results to marine fish should be done with caution and highlights the need for future studies on marine fish.

3. Marine fish and cyanide

As discussed above, several studies have shown that cyanide toxicokinetics is very species dependent. This reiterates the 2008 NOAA Cyanide workshop recommendation for studies on cyanide specifically in marine fish and ideally in coral reef species.

Some of the coral reef species targeted for the live fish for food and marine aquarium trade include:

- **For food trade:** Large groupers, coral trout, barramundi cod and humphead wrasse (Bruckner and Roberts, 2008).
- **For aquarium trade:** Damselfish (*Pomacentridae*) make up almost half of the trade, with species of angelfish (*Pomacanthidae*), surgeonfish (*Acanthuridae*), wrasses (*Labridae*), gobies (*Gobiidae*) and butterflyfish (*Chaetodontidae*) accounting for approximately another 25-30 percent. The most traded species are the blue-green damselfish (*Chromis viridis*), the clown anemonefish (*Amphiprion ocellaris*), the whitetail dascyllus (*Dascyllus aruanus*), the sapphire devil (*Chrysiptera cyanea*) and the threespot dascyllus (*Dascyllus trimaculatus*) (Wabnitz et al, 2003), emperor angel-fish (*Pomacanthus imperator*), blue surgeon-fish (*Paracanthurus sp*) and blue ring anglefish (*Pomacanthus annularis*) (70-80 percent from Indonesia and Philippines, were cyanide is most prevalent) (Bruckner and Roberts, 2008).

The 2008 NOAA Cyanide workshop focused on the requirement to determine the detectable level of cyanide and cyanide metabolites in coral reef fishes and to understand how these levels change over time following exposure. It recognised that experiments at varying life history stages should be conducted to investigate how levels change over time following exposure at multiple concentrations (Bruckner and Roberts, 2008). Nine years on from the workshop, there is still very little information available on the topic.

There are three key studies of exposure to cyanide in marine reef fish which investigate the evolution of levels with time elapsed following exposure. In 1998, Hawana et al conducted experiments exposing the Humbug damselfish *Dascyllus aruanus* to 25 and 50 mg/L of cyanide under different conditions and different exposure times. Exposure for a period of 120 seconds or a period of stressed conditions resulted in a lethal effect at both concentrations. In 2012, Vaz et al. conducted experiments exposing clown fish (*Amphiprion clarkii*) to concentrations of 12.5 and 25 mg/L of cyanide for 60 seconds. They measured the SCN^- concentration in water after 28 days which reached up to 6.96 µg/L of SCN^- for the 12.5 mg/L experiments and 9.84 µg/L of SCN^- for the 25 mg/L experiments. Levels of SCN^- for the lowest exposure concentrations only appeared from day 6 and levels for both

concentrations was reported to be stable from day 21 to 28 (Figure 6). The authors recorded mortality for both concentrations during exposure but final survival rates were 89 percent and 67 percent respectively. However, it is estimated that the overall mortality for cyanide-caught fish from reef to retailer exceeds 90 percent (Rubec et al., 2001). Data from Vaz et al. suggests that cyanide can stay in fish tissues for up to 3 weeks supporting Rubec et al.'s (2003) observation that clown fish do not quickly metabolize cyanide to SCN^-, and therefore SCN^- is excreted in the days following exposure rather than the first few hours. Importantly, Vaz et al. (2012) provides an indication that SCN^- concentration in fish holding tanks can be expected to be in the lower µg/L.

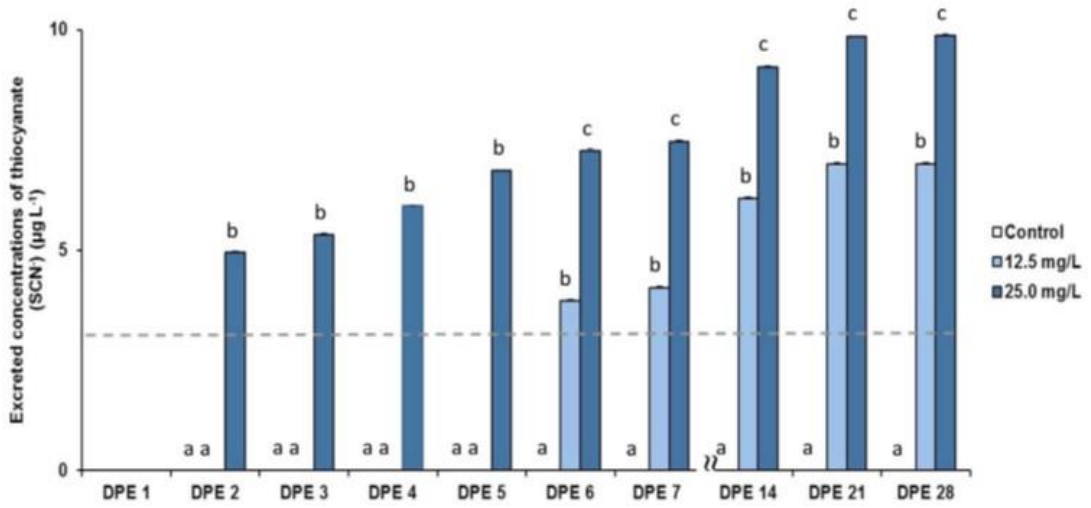


Figure 6. Concentrations (µg/L) of thiocyanate excreted during the depuration period of Amaphiprion clarkii. DPE: Day post exposure (Vaz et al., 2012).

Andrade (2015) exposed 60 small and 60 medium sized clown fish (*Amphiprion ocellaris*) to different concentrations of cyanide (6.25, 12.5, 25, 50 and 100 mg/L) for 60 seconds in synthetic seawater. Fish behaviour was monitored during exposure and for 30 minutes after exposure. Ninety six hours after exposure to cyanide, fish were assessed for biomarkers. All small fish showed loss of respiratory activity and sank to the bottom of the tank after exposure to all concentrations (6.25, 12.5, 25, 50 and 100 mg/L). The time of recovery increased with increasing concentrations with 100 percent survival at the lowest concentration, decreasing to 70, 40, 20 and 0 percent with the increasing concentration. For the medium sized fish, survival was 100 percent for 6.25, 12.5 and 25 mg/L, 50 percent for 50 mg/L and 0 percent for 100 mg/L. The study confirmed that vulnerability to cyanide poisoning may vary intraspecifically (e.g., specimen size) and interspecifically (even in species which are closely related phylogenetically). The clownfish *A. ocellaris* displayed the highest tolerance to cyanide poisoning compared to other studies with pomacentrids

(Andrade, 2015). The levels of cyanide the fish are exposed to during collection is reported to be as low as 6.25 mg/L, enough to affect behaviour and allow their easy capture.

KEY POINTS:

There are three key scientific studies which investigate the exposure of coral reef fish to cyanide; Hawana et al., 1998; Vaz et al., 2012 and Andrade, 2015.

- It is reported that 6.25 mg/L of CN exposure (the lowest tested) has effects on fish behaviour. Exposures to 12.5mg/L of CN started to show a reduction in survival (70 %) while exposures for 60 or more seconds to 25 mg/L of CN- or higher showed a reduction in survival from 60 % to 100 %, especially after stress.
- SCN- was detectable in artificial seawater from 6 days post exposure for the lowest cocenctration and was at stable levels from day 21 to 28.
- Differences between species and different sized fish from the same species were recorded reiterating the importance of targeted studies in relevant species.
- Future work should be designed to understand the behaviour of different compounds in different fish species but also to discriminate background levels due to biological production or environmental exposure from levels present following cyanide fishing.

4. Methods of detection

The original method used for the analysis of cyanide in marine fish on a large scale was cyanide-ion selective electrodes (ISE). This method was applied by the network of Cyanide Detection Laboratories (CDL) established in the Philippines in their Cyanide Detection Test (CDT) programme. Results using this method showed ~40 percent of positives in samples, even in fish up to 5-14 days after exposure, and negative results in non-cyanide caught fish (Rubec et al., 2003). The method had the advantage of having the personnel and infrastructure in place so it was the preferred method in the countries of export (Bruckner and Roberts, 2008). However, the method was challenged because erroneous results were observed due to interferences with the ISE electrode. The precision of the technique was also challenged due to the nature of cyanide which can transform into thiocyanate and other metabolites very quickly, and can thus be at non-detectable levels on arrival at collection and export points. Cyanide may also be oxidized by sulphuric acid during digestion and the original method was not verified with a secondary recognised method nor were the recoveries in biota samples tested (Bruckner and Roberts, 2008). The questions that remain about the reliability of methods for detecting cyanide highlight the importance of selecting the appropriate target compound, the most suitable matrix/sample to perform the analysis and the technique which will give the most accurate and reliable results.

4.1 Considerations for different target compounds

4.1.1 Cyanide (CN-)

Cyanide can be analysed using spectrophotometric and fluorescent detectors, both of which require derivatization steps and extraction to eliminate interferences. In some cases, HCN analysis requires a pre-treatment to liberate the bounded-HCN, especially for the analysis of blood samples. Because cyanide can bind to a number of blood components (proteins, haemoglobin, etc.) and the total cyanide is made up of both free and bound cyanide, the ideal method should have access to both (Jackson and Logue, 2017). The initial spectrophotometric methods usually require the reaction with an aromatic amine to produce detectable products. However, these products are unstable and they lack specificity due to interferences from another chemical species, especially thiocyanate and thiosulphate. Fluorescence methods are available with no thiosulfate interference and with higher sensitivity than the spectrophotometric methods. The influence of interferences can be reduced using a separation technique like liquid or gas chromatography with both spectrophotometric and fluorescent detectors. Cyanide can also be analysed with biosensors based on microbial sensors, enzyme inhibition and degradation. As cyanide is

volatile, gas chromatography (GC) can be used without derivatization and only requires a way of trapping the HCN using a headspace or cryogenic trap. Derivatization is needed for analysis by GC with electron capture detector (ECD). However, the rapid increase in levels of HCN after exposure and the levels detectable make it a main target for quick portable devices. Its use is limited by the time elapsed after exposure (Logue et al., 2010, Jackson and Logue, 2017).

4.1.2 Thiocyanate (SCN-)

Thiocyanate is weakly spectrophotometrically active so it does require derivatization. Cyanide and thiocyanate have been analysed together by GC with nitrogen-phosphorous detector (NPD) which also requires derivatization. They have also been analysed together using reverse phase liquid chromatography associated to spectrophotometric and fluorometric detectors. Cyanide and thiocyanate have been analysed using ion-exchange chromatography using ion selective electrodes (ISE) and spectrophotometric detection. Gas chromatography has also been used for the analysis of cyanate, thiocyanate and ATCA using electron capture detector (ECD), nitrogen-phosphorous detection (NPD) and mass spectrometry (MS). Thiocyanate and ATCA are not volatile, so they need to be derivatized which adds an additional analytical step. The levels of SCN^- after exposure are high compared to other metabolization products making it a good target compound for portable devices (Logue et al., 2010, Jackson and Logue, 2017). Biosensors have currently not been used for the analysis of SCN^- (Logue et al., 2010).

4.1.3 ATCA

ATCA usually needs pre-treatments to separate it from components of biological samples. It has been analysed using spectrophotometric techniques but requires derivatization to produce a coloured product. Limitations to this method are long sample preparation and the interference with other species including cyanide ions. ATCA has also been analysed by RP-HPLC with fluorometric detection which does not require derivatization. Because ATCA is not volatile, analysis by GC-MS and GC-ECD does require the additional step of derivatization. (Logue et al., 2010).

4.1.4 Cyanide-protein adducts

Cyanide-protein adducts analysis involves the isolation of the protein of interest with subsequent enzymatic digestion. This is usually a very time-consuming process and needs powerful instrumentation. They have been analysed using RP-HPLC and fluorometric detection (Logue et al., 2010).

KEY POINTS:

- Due to the low stability and short life of cyanide, and the low abundance of other metabolites (of which our understanding is still lacking), thiocyanate (SCN^-) has been selected as the main target compound for assessment in this report. Biological samples have been discarded here due to limited quantities of material (blood, saliva, urine) for analysis in fish, therefore, exisiting methods which analyse thiocyanate in liquid or aqueous samples have been selected for this assessment.

4.2 Considerations for different samples

Most published methods for the determination of cyanide exposure focus on analysis for exposure studies in humans andin particular blood, urine, saliva, expired air and tissue samples. For fish, most of these matrices can be difficult to obtain due to the size of the fish. In addition, the rates of cyanide metabolism may be inconsistent between organs: the enzyme that catalyses the conversion of cyanide to thiocyanate has highly variable concentration depending on the organ, therefore, the selection of the analyte to be determined and the tissue to be sampled are extremely important (Logue et al., 2010).

Due to the quick metabolization of cyanide, CN^- can only be analysed in tissues; excreted CN^- will be in the form of metabolites such as SCN^- and ATCA. This rapid detoxification of cyanide means that samples for cyanide analysis should be collected as quickly as possible following exposure. If analysis has to be delayed and samples have to be stored, the variation of original cyanide levels due to evaporation, oxidation, formation of adducts with proteins and formation from other compounds should be considered (Logue et al., 2010). Another consideration is that the ideal method for analysing cyanide exposure to marine fish is that it is non-intrusive and non-destructive given the fact the fish are traded live for the aquarium trade or for the live fish for food trade. This reduces the type of matrix that can be analysed: tissue is therefore discarded. Blood could be obtained but most probably in very low quantities, so a method based on the analysis of blood would need to be very sensitive. Aqueous and urine related methods are therefore considered to be the most suitable for this report on live marine fish.

Careful sample preparation and storage of biological samples for the analysis of cyanide or associated metabolites is critical to produce accurate results. Interconversion between cyanide and thiocyanate is the main problem for the analysis of these compounds. It can happen during sample preparation and storage. Low temperatures, high pH and the addition of preservative

agents are common procedures to prevent the loss of cyanide. Low temperatures are extremely important to reduce evaporative loss and slow biochemical reactions (Logue et al., 2010).

4.3 Considerations for different analytical techniques

There are a number of review papers which focus on alternative methods for the analysis of cyanide and metabolites. Most of these studies focus on the analysis of cyanide itself and only a few address the analysis of thiocyanate or cyanide metabolites. In 2004, Lindsay et al. reviewed the methods for the analysis of cyanide in blood and in 2005, Mak et al. produced a review of biosensors for the analysis of cyanide. Later, Ma and Dasgupta (2010) reviewed developments in cyanide detection, which was updated by Randviir and Banks in 2015 with developments in quantifying cyanide and hydrogen cyanide. In 2017, Jackson and Logue reviewed the latest rapid and portable techniques for the diagnosis of cyanide exposure. To date, the only review that focuses on the analysis of cyanide metabolites (thiocyanate, ATCA and cyanide adducts) is that by Logue et al. (2010). Different techniques and analytical methods have different limitations depending on the matrix and the target compound considered. These are summarised in Figure 7.

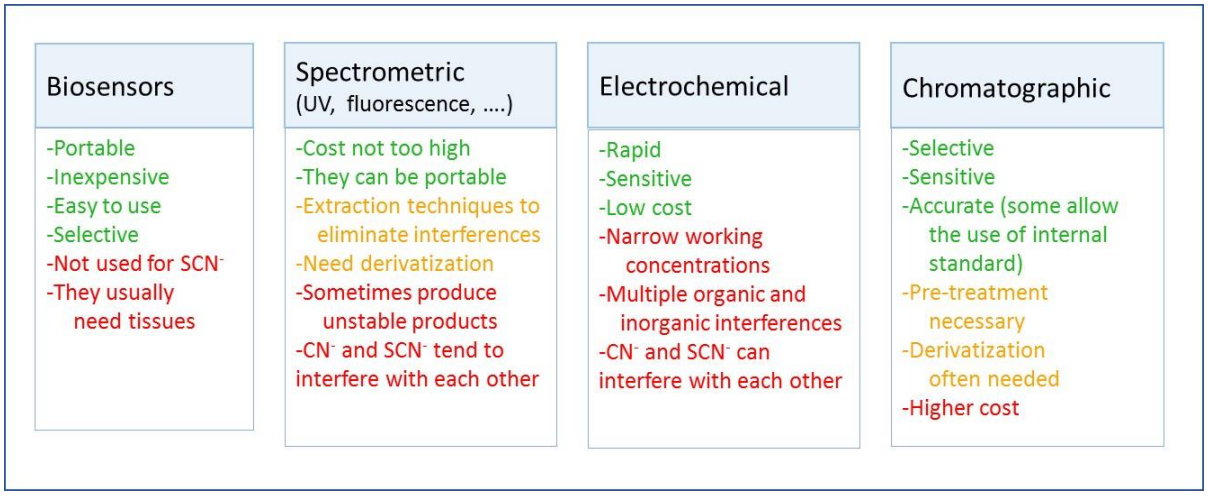


Figure 7. Summary of key characteristics of different techniques including the main advantages (green) and challenges (orange and red).

4.3.1 Biosensors

Biosensors are portable, inexpensive, easy to use and can have high selectivity but have to date not been used for the analysis of SCN^- (Logue et al., 2010). However, they can be slow, suffer from degradation of the biological components, have inconsistent electrochemical signals and produce low levels of the active sensor detection form (Bruckner and Roberts, 2008; Logue et al. 2010). In 2005, Mak et al. produced a review (following a

PhD thesis on the topic) relating to biosensors for the detection of cyanide, covering microbial, inhibiting-enzyme and degrading-enzyme biosensors. Mak et al. concluded that they should all be discarded for a number of reasons. The microbial sensors are suitable for environmental analysis because they are inexpensive and simple to produce and operate but they are not selective. The biosensors based on enzyme inhibition are not ideal because the enzymes need to be regenerated or replaced, and the number of natural enzymes which could be used are very limited. While the sensors based on cyanide degradation would be ideal because they are very selective, they lack sensitivity. The authors suggested a biosensor in which cyanide degrading enzymes are added to a formate biosensor would provide a viable alternative, but this option would require the fish to be sacrificed.

A more recent study by Andrade (2015) also considered biomarkers for detecting cyanide exposure in clown fish (*Amphiprion ocellaris*), a popular ornamental species. Andrade exposed small and medium sized fish to different concentrations of cyanide as described in section 3 *Marine fish and cyanide*. They found that the induction levels of activity for the enzyme catalase in liver, and the activity for the enzyme glutathione S-transferases (GST) in muscle, had a relationship with the exposure concentrations to cyanide. Other biomarkers such as lipid peroxidation (LPO), glutathione peroxidase (GPx) or acetylcholinesterase (AChE) were discarded for not showing a clear relationship or varying activity between different size fishes. Again, fish needed to be sacrificed for these experiments therefore, biosensors are not a suitable technique for live fish.

4.3.2 Spectrometric and fluorescent techniques

Spectrometric and fluorescent detectors require derivatization steps, for the analysis of blood they also need extraction techniques to eliminate interferences. Spectrometric methods sometimes produce unstable products and the cyanate forms (CN^-, SCN^-) interfere with each other. Fluorescence methods are available with no thiosulfate interference and with higher sensitivity compared to the spectrophotometric methods. (Bruckner and Roberts, 2008; Logue et al. 2010). The main problem with these techniques is the lack of specificity, which means that they potentially are prone to interferences. Studies are required to ensure the method would be suitable for the analysis of seawater samples.

4.3.3 Electrochemical techniques

Electrochemical methods are available for the analysis of cyanide and thiocyanate with high sensitivity and rapid analysis time. However, they can be subject to multiple organic and inorganic interferences, including S^{2-}, ClO_4^-, NO_2^-, N_3^- and I^-, narrow working concentrations and large sample sizes. Polymeric membrane-based methods (ISEs) have been developed

to address some of the issues associated with electrochemical methods. They exhibit rapid response, high sensitivity, wide linear range, low cost and are simple to operate. However, the issues with the interaction of multiple ions and biological materials present in biological samples means that they have seldomly been used for analysis of biological samples. This problem can be alleviated by removing interferences with the addition of chemicals, as is done for the ISE method from the American Society of Testing and Materials (ASTM) which is used for the detection of cyanide in fish (Bruckner and Roberts, 2008; Logue et al. 2010). However, inaccurate results have been reported with this method, suggesting that the method is susceptible to interferences from marine samples.

4.3.4 Chromatographic techniques

Complexity associated with matrices have led to an increase in the use of chromatographic techniques to separate cyanide and its markers in biological samples from interferences. Liquid chromatography (LC) has been used with reverse phase and ion chromatography columns. Reverse phase usually requires pre-treatment steps. LC has been used with fluorometric, spectrometric and mass spectrometry detectors. Gas chromatography has been used for cyanide, thiocyanate and ACTA determination, although thiocyanate and ACTA are not volatile enough and need an additional derivatization step. Electron capture (ECD), nitrogen-phosphorous (NPD) and mass spectrometry (MS) detectors have been used for their analysis, although NPD can be unstable at times and is not as sensitive as the other detectors. GC-MS analysis is very sensitive and allows the use of stable isotope internal standards (^{13}CN, $C^{15}N$, $^{13}C^{15}N$), which can be used to correct matrix effects (Logue et al., 2010).

It is important to note that there has been a great number of studies relating to the development of new sensors for the detection of cyanide in the last few years, albeit with few focusing specifically on cyanide products. However, most of these sensors are still the in the R&D stage. Wang et al. (2014) and Randviir and Banks (2015) reviewed some of the newly developed sensors. It should also be noted that there is an increment in colorimetric techniques which allow the visual detection of cyanide. Zelder and Mannel-Croisse (2009) produced a review of colorimetric techniques, which were also included in the reviews from Ma et al. (2010), Randviir et al. (2015) and Jackson and Logue (2017).

KEY POINTS:

- When selecting a methodology to detect the use of cyanide fishing, the target compound (CN, SCN^- etc), the matrix/sample (blood, urine etc), and the analytical technique used are important considerations and often interdependent.
- Analysis of most of target compounds require pre-treatment steps to transform them into volatile (ex. to analyse using GC) or visible forms (ex. for UV or fluorescent detection).
- Care should be taken when treating and storing the sample to avoid chemical reactions and interconversions which will lead to inaccurate results. Some techniques are more susceptible to interferences than others (have low selectivity) (e.g., electrochemical techniques, spectrometry), and some have higher sensitivity to the target compound (e.g., fluorescence, mass spectrometry).

4.4 Assessment of available methods

4.4.1 Method of assessment

Most available scientific studies that analyse cyanide and its metabolites in biological sample matrices are for free cyanide which can potentially only be measured in the hours immediately following exposure and in biological tissues or fluids. Therefore, our review and assessment focuses on non-destructive methods for analysing thiocyanate (SCN^-) or other cyanide metabolites, and specifically for aqueous and liquid samples. The studies that have been reviewed are summarized in Table 1, Annex 1. Priority was given to papers describing methods applied to samples rather than those describing new sensors or techniques which have not been applied to samples and therefore tested operationally. However, a number of studies have described the possibility of analysing SCN^- after oxidising it to CN^- (Paul & Smith 2006; Al-Saidi et al., 2016), therefore a second table has been compiled of methods developed, mainly for blood analysis, but which could be easily applied to water samples or transformed into portable equipment (Table 2, Annex 2).

The papers summarized in Table 1 and 2 (Annex 1 and 2) have been selected from studies on the analysis of thiocyanate and cyanide metabolites in the key review papers by Logue et al. (2010), Randviir and Banks (2015) and Jackson and Logue (2017) and from the literature published in recent years (2010-2017). To ensure the quality and detail of the method required for an adequate evaluation, we have focused on relevant journals in the field of analytical chemistry.

For the systematic evaluation of the studies reviewed, we have followed the categories proposed by Klimisch et al. (1997) with modifications to adapt the approach to analytical criteria. We have applied the following scoring scheme to each method reviewed based on the detail provided and expert judgement:

1: Reliable without restriction,

2: Reliable with restriction,

3: Not reliable,

4: Not assignable.

Papers were scored as 1 when all the analytical quality criteria (recoveries, limits of detection, linearity, validation of the method, interferences) were presented with satisfactory experimental results. Category 2 was allocated to methods that have recorded good quality criteria but incomplete information. Category 3 was allocated to methods lacking significant information which meant that the quality of the method was difficult to evaluate, or with some unsatisfactory experimental results (e.g., poor recoveries). Category 4 was assigned to methods that could not be assigned to any other category because there is insufficient data from a reliable source for assessment.

4.4.2 Results of the assessment

Tables 1 and 2 (Annex 1, 2) summarize the main characteristics and data quality for each of the selected papers to which a score based on the Klimisch system was assigned. As previously stated, studies were selected for assessment which focus on liquid or aqueous matrices which are potentially easier to apply to seawater, and on those which analyses thiocyanate and other metabolites since the half-life of cyanide is too short to be detected in import countries. Colorimetric techniques have also been assessed as they offer great potential for the analysis of cyanide in the field.

4.4.2.1 Detection of thiocyanate

One key study for the detection of thiocyanate is that by Vaz et al. (2012), who applied a fiber optic detector to synthetic seawater holding live fish. This method had no sample preparation and claimed to be able to detect SCN^- at levels as low as 3.16 µg/L. Its main advantage is that it has the potential to be a field method as it is a rapid and non-intrusive method, although portability would likely entail miniaturization of the system. The authors based their study on the method developed by Silva et al. (2011) who reported a very similar limit of detection (3 µg SCN^-/L) after a minimal preparation step of filtrating the water samples. Silva et al. analysed natural (rather than synthetic) seawater and reported the lowest levels of 7.2 µg of SCN^-/L in water from Barra (Portugal).

Herz et al. (2016) tried to replicate the work by Vaz et al (2012) in natural seawater samples but could not detect SCN^-. They suggested that natural seawater is too complex to be analyzed using the method described by Vaz et al. (2012) and that there were too many parameters to make it suitable for the analysis of SCN^-. However, Herz et al. (2016) did not specify some important technical information, including the detection technique used (which was bespoke in Vaz et al. 2012), necessary for a critical comparison of these two studies. In summary, further experimental evidence is required on the application of the methods developed by Silva et al. (2011) and Vaz et al. (2012) in natural seawater samples.

Promising laboratory-based methods using liquid chromatography with a UV detector include that of Rong et al. (2005). They analyzed natural seawater samples after a simple filtration step and reported recoveries for SCN^- of 102-106 percent and limits of detection of 2 µg/L. They further applied their method to the analysis of seawater samples from Japan and detected levels of SCN^- as low as 8.1 µg/L in the samples from Okinawa. Al-Saidi et al. (2016) also used a UV detector and head space for the detection of SCN^- in waste water and saliva after transforming the SCN^- into CN^-. They reported low limits of detection of 9 µg/L, but the head-space extraction system they used was bespokely made which adds to the risk of losing cyanide if samples are not correctly managed.

Jafari et al. (2010) used negative electrospray ion mobility spectrometry for the analysis of SCN^- in saliva. They only used the simple steps of a centrifuge and a sample dilution and reported a limit of detection of 3 µg/L. This technique has the potential to be portable as it is simple, rapid and requires no sample treatment. However, the main limitation is that the instrument was a custom-made unit including an ion mobility spectrometer cell, the electrospray needle, two high voltage power supplies, a pulse generator, an analog to digital converter and a computer so is considered to be in the R&D domain. It was also used for saliva, so its applicability to seawater would have to be studied further.

Another method that has attracted attention after a presentation in the 2016 International Aquarium Congress in Canada is the one by Dashti (2016). Dashti claimed to be able to detect less than 5 ng/L of SCN^- in seawater (which is 1000 orders of magnitude lower than the detection levels discussed above) using pre-concentration of the sample and a detection system based on the use of gold and a portable spectroscopic device (Surface enhanced Raman scattering detector or SERS). Interestingly, this method uses portable SERS units which are commercially available. However, no additional information was given in the presentation and the data has not been published in the peer-reviewed literature, so it is not possible to fully evaluate the method in this assessment.

One of the most popular substrates for SERS is gold nanoparticles which are very stable and produce enhancement in many SERS systems (Pienpinijtham et al., 2011). There are two studies in the literature analyzing SCN^- using SERS and gold nanoparticles. One is by Pienpinijtham et al. (2011) who developed a method which uses gold nanoparticles using starch as reducing agent and stabilizer. Using this method, the authors reported a limit of detection of 0.05 µM (2.9 µg/L) with a linear range of 0.05 to 50 µM. They found no significant interferences from other anions such as other halides, carbonate and sulfate, but cyanide (CN^-) was seen to damage the gold surface and interfere with the analysis of SCN^-. Hou et al. (2017) used magnetic sheets with gold nanoparticles, applying their method to the analysis of SCN^- in milk and saliva. They reported a possible limit of detection of 10 ng/L, with recoveries of 95-112 percent and RSDs lower than 10 percent. Both methods have the potential to be portable.

Song et al. (2015) also used gold nanoparticles combined with fluorescein and a spectrofluorometer for the analysis of SCN^- in milk and saliva. Saliva samples were simply centrifuged, filtered and diluted making the analysis of water samples promising, although it is likely that some optimization would be required. They reported recoveries of 100-108 percent and limits of detection of 0.09 nM (5 ng/L), the most sensitive method found in the literature reviewed. However, this approach has not been reported elsewhere and remains in the R&D domain. Additionally, most of these authors since have reported a different approach to SCN^- determination in milk using gold nanoparticles and colorimetry (Song et al., 2016); a method that is over 70 times less sensitive (0.38 µg/L) than reported in their 2015 paper.

Another method for the detection of thiocyanate, attracting attention due to its potential portability and low sensitivity (2 µg/L) in preliminary trials with untreated marine water, is that provided by Murphy and Rhyne (2017). This study explored the use of meso-tetratolylporphyrins (iron (III), ruthenium (III), zinc (II)) in an electrode, and they characterized it by cyclic voltammetry and UV-Vis absorption spectroscopy. These electrodes are used in the development of a portable device to detect thiocyanate. As with other newly developed sensors, these are not commercially available. However, no additional information was provided in their media release and the underlying data has not been published in the peer-reviewed literature so it is therefore not possible to fully evaluate the method in this assessment.

Gas chromatography (GC) has been used for the determination of SCN^-, allowing the separation of thiocyanate and other metabolites from interferences, and therefore providing a more selective technique than others. However, a derivatization step is needed due to the

lack of volatility of cyanide metabolites. Bhandari et al. (2012, 2014) used GC with electron capture negative ionization mass spectrometry (ECNI-MS) for the analysis of SCN^- and cyanide in blood and plasma. The complexity of the biological matrix required the use of an extraction step which might be omitted for water samples. They reported recoveries >90 percent and limits of detection of 50 nM (2.9 µg/L) for SCN^- and 1 µM (26 µg/L) for CN^-.

Abdolmohammad-Zadeh and Vasli (2014) also used GC with a flame ionization detector (FID) for the analysis of SCN^- in saliva and serum. In this study, the extraction of the sample was based on solid-phase extraction using nickel-aluminum layered nanosorbent with higher reported recoveries (94 percent) and limits of detection of 0.2 µg/L. Interestingly, Ammazzini et al (2015) developed a GC-MS methodology with minimum sample preparation using headspace sampling with a simple, single-step derivatization of SCN^- in the sample (saliva), achieving limits of detection expected to be well below 5 µg SCN^-/L. Amongst the gas chromatographic methods reviewed, this latter method is of particular interest as a lower cost, relatively rapid method which provides high SCN^- specificity and analytical accuracy (through the use of isotopically labelled surrogate standards). Mass spectrometry was also used by Nakamura et al. (2015) coupled to an ion chromatograph for the analysis of SCN- in blood. They used only 25 µg of blood, but in an extraction system that used an in-house micro ion extractor. The recoveries they reported were very low, between 31 and 43 percent, which could also explain the relatively high limit of detection of this method of 29 µg/L.

A completely different approach was taken by Pena-Pereira et al. (2016). They analyzed SCN^- using a method based on the iron (III)-thiocyanate colored complex in a paper-based sensing platform, followed by image analysis using a scanner as detection device (Figure 8). They validated this method with a spectrophotometric method for the analysis of 2 µL of saliva, and reported recoveries between 96 and 104 percent, and a limit of detection of 0.06 mM (3,500 µg/L). Although this method is extremely simple and completely portable, its limit of detection is expected to be much too high for the analysis of SCN^- in environmental samples which is in the low µg/L.

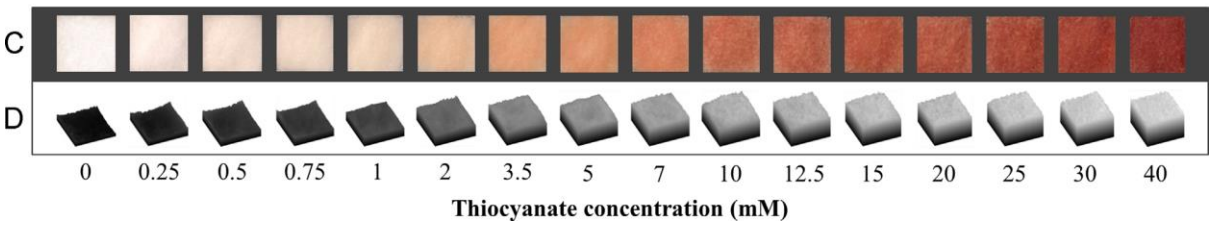


Figure 8. Scanometric images of a paper-based device spotted with increasing concentrations of thiocyanate (C) and the corresponding surface plots obtained by processing them by ImageJ using the blue channel (D) (Pena-Pereira et al., 2016).

4.4.2.2 Detection of other by-products

Alternatives to the analysis of SCN^- include the analysis of other cyanide metabolites such as the α-KgCN (α-ketoglutarate cyanohydrin), the protein bound-cyanide and ATCA (end of Table 1 in Annex 1). There are only three studies which address the first two metabolites. Mitchell et al. (2013b) developed a method for the analysis of α-KgCN in plasma using UPLC-MSMS, reporting a limit of detection of 200 nM (37 µg/L) and very low recoveries (14-27 percent). While Youso et al. (2010, 2012) used GC-MS for the analysis of protein-bound cyanide in plasma, recording limits of detection of 2.5 µg/L or 40 nM, however no recoveries were reported.

The most realistic alternative to SCN^- seems to be the ATCA. Several studies have developed methods for the analysis of ATCA, mainly in blood or plasma. Most of these methods originate from the Logue research lab and seem to require derivatization. Logue et al. (2005) developed a method for plasma using Solid Phase Extraction (SPE) and derivatization for the analysis of ATCA by GC-MS. Their recoveries were between 84 and 100 percent, with a limit of detection of 25 µg/L. Lulinksi et al. (2015) used HPLC-MSMS for the analysis of ATCA in blood. They used imprinted polymers for the extraction of SCN^- and compared it to other commercial polymers used in other methods. The authors saw a reduction in interferences compared to other sorbents and an increase in sensitivity, reporting recoveries of 81-89 percent and limits of detection of 3.5 µg/L. Giebułtowicz et al. (2016) also developed a method for the analysis of ATCA in blood based on the precipitation and extraction of ATCA (and derivatization) for the subsequent analysis using HPLC-MSMS. They reported recoveries of 86-107 percent and limits of detection of 0.43 µg/L.

4.4.2.3 Colorimetric methods for cyanide

Analysis of cyanide using colorimetric methods have been selected for assessment here due to their potential in the application of field screening. Some of these approaches use visual detection (“naked eye”), while others require instrumental detection (mainly spectrophotometric and fluorometric) allowing a quick quantitative analysis in addition to the qualitative analysis. Cyanide levels that produce a colorimetric change visible to the human eye would allow a qualitative or semi-quantitative analysis. Below that level, only instrumental methods can confirm the presence of cyanide in samples, and only instrumental detection would allow accurate quantitative analysis.

Several authors use spectrophotometric detectors for the analysis of cyanide using the reaction of cyanide with different compounds to generate products that have colorimetric properties (chemosensors).

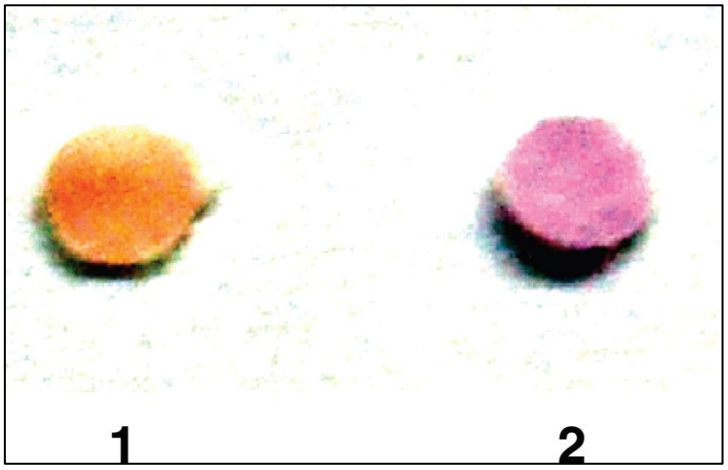


Figure 9. Qualitative cyanide assay using a cobinamide-saturated glass fibre filter. (1) Left filter is from a rabbit RBC sample containing no cyanide. (2) Right filter is from a rabbit RBC sample containing 15 µM cyanide (Blackledge et al., 2010).

Blackledge et al. (2010) used cobinamide for the detection of cyanide in blood and plasma using UV-visible, reporting a limit of detection of 0.25 nmol (< 1µM [58.4 µg/L]). Their method also covers the possibility of a qualitative mode by visually observing the colour change in cobinamide-impregnated paper for cyanide concentrations >30 µM (1752 µg/L) (Figure 9). Cobinamide has also been used by Tian et al. (2013) for the development of a device that analyses volatilized cyanide, isolating it from the matrix. Their field method using a spectrophotometer (Figure 10) allowed them to detect 2.2 µmol/L (129 µg/L).

Figure 10. Qualitative cyanide assay using cobinamide using increasing concentrations of cyanide (Tian et al., 2013; Figure from Jackson and Mitchell, 2017).

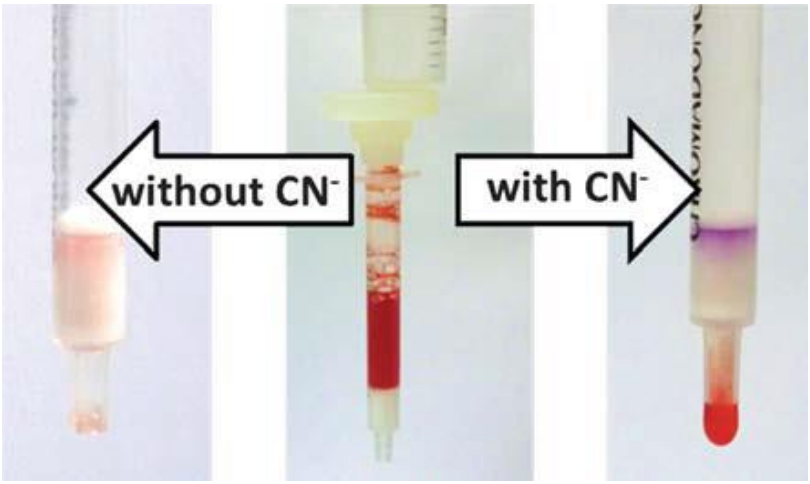


Figure 11. Image of the experimental set-up for the detection of blood cyanide with chemosensor. Comparison of the C18ec column upon passing blood without cyanide (left) and with spiked cyanide (CN- 40 mM [2336 mg/L]; right) (Mannel-Croise and Zelder, 2012).

Mannel-Croise and Zelder (2012) used a corrin derivative as a chemosensor for the analysis of cyanide in blood using UV-vis spectroscopy and DRUV-vis spectroscopy in a hand-held detector. The chemosensor was adsorbed on the top of a SPE cartridge (Figure 11) while in a previous studywith cassava (Mannel-Croise at al. 2009), the chemosensor was applied directly to the samples. The limit of detection they reported in the SPE method was 3 µM (175 µg/L) for both the DRU-vis and the "naked-eye" methods. This method was then modified by Zelder and Tivana (2015) to analyze cyanide in cassava samples. In that particular case, the authors reported the requirement to add an enzyme to ensure all cyanogens were converted to cyanide before the detection. This was achieved using the enzyme from the cassava leaves (Figure 12).

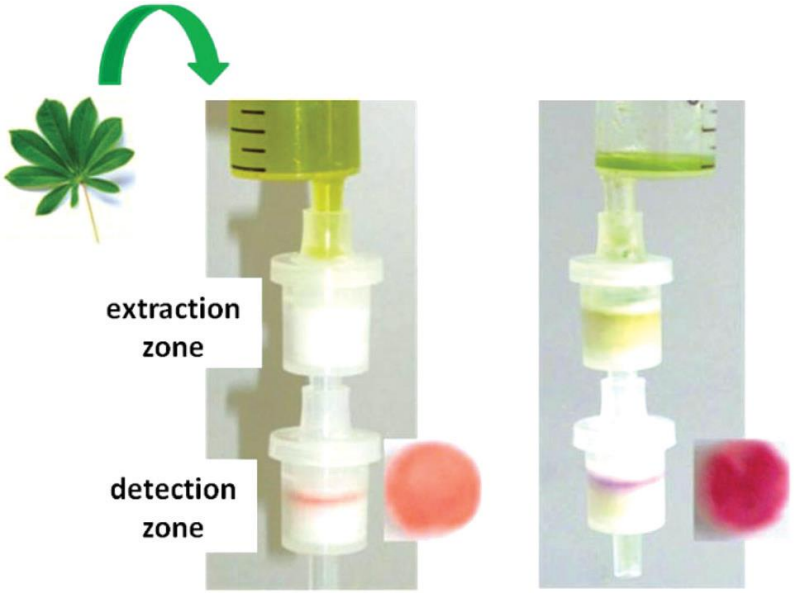


Figure 12. Qualitative cyanide assay using cobinamide using increasing concentrations of cyanide (Zelder and Tivana 2015).

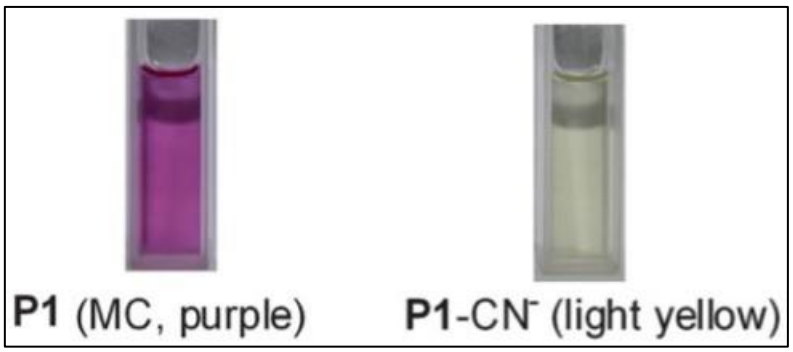


Figure 13. A spirooxazine derivative 2-nitro-5a-(2-(4-dimethylaminophenyl)-ethylene)-6,6-dimethyl-5a,6-dihydro-12H-indolo[2,1-b][1,3]benzooxazine (P1) as a sensitive cyanide indicator (Zhu et al., 2012).

Zhu et al. (2012) used UV-visible spectrophotometry for the analysis of cyanide using as chemosensor a spirooxanzine derivative reporting a limit of detection of 0.4 µM (23.4 µg/L). They reported some interferences from the sulphide (S2-) (Figure 13).

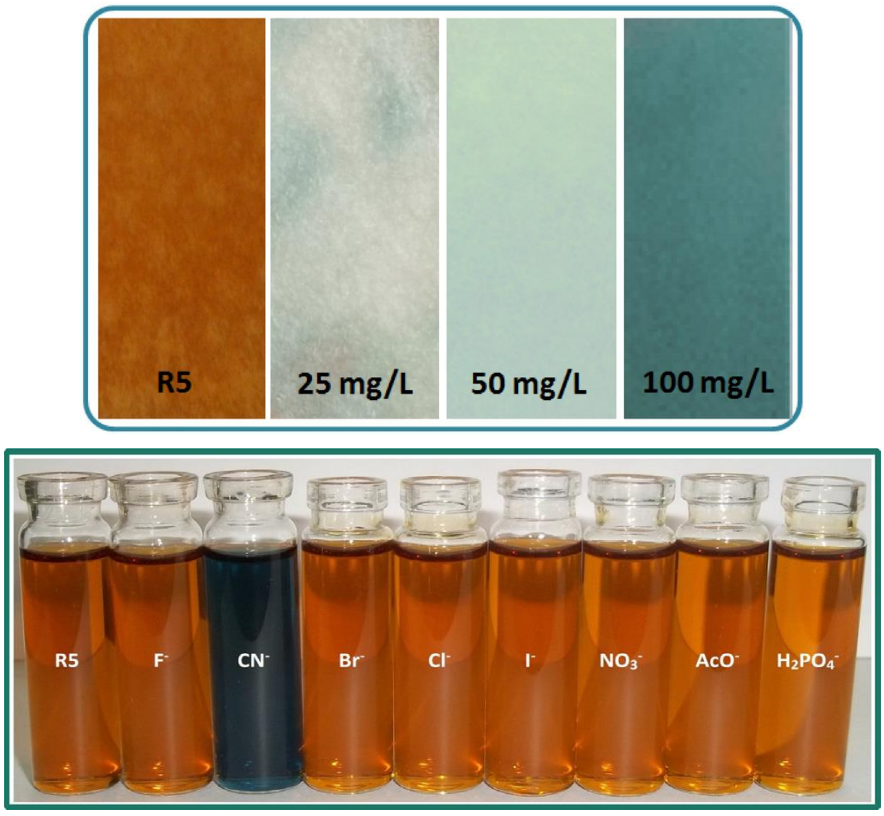


Figure 14. Colour changes observed in aq. HEPES buffer:DMF (1:1 v/v) solution of Receptor 5 upon addition of various anions and Colour changes of test strips upon dipping in cyanide ion solution in deepwell water (Jayasudha et al. 2017).

Jayasudha et al. (2017) developed a method based on naphtohinone-indole base receptors which are selective to cyanide ion in aqueous HEPES buffer with DMF detecting 0.01 – 2.1 nM (0.58-123 ng/L). The sensing abilities of the chemosensors were investigated by "naked eye" experiments, which exhibited a change in colour to blueish in pH 7.2 so are

suitable for sensing in real-life samples. Test strips were used to sense CN^- ion of different concentrations in well water (Figure 14).

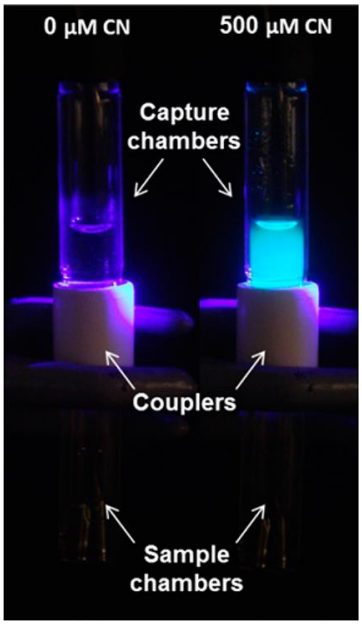


Figure 15. Field sensor for the diagnosis of cyanide exposure was developed based on the reaction of naphthalene dialdehyde, taurine, and cyanide, yielding a fluorescent β-isoindole (Jackson et al. 2014).

Jackson et al. (2014) developed a method using naphthalene dialdehyde and taurine which form a fluorescent product once linked to cyanide (Figure 15). They developed a bespoke mechanism to extract the cyanide from blood samples for analysis in the field and reported a limit of detection of 0.78 µM (45.6 µg/L).

Figure 16. Photographs of P1 ([RU] = 10 _M) inTHF/H2O (98:2, v/v) with CN−ranging from 0, 1, 5, 10, 20, 30, 40, 50, 80, 100, 150, 200 and 300 µM from left to right under UV excitation (365 nm) (b) and visually (c) (Wang et al. 2015) (Jackson et al. 2014).

Wang et al. (2015) used a BODIPY dye derivative (4-difluoro-4-bora-3a,4a-diaza-s-indacene) and fluorene for the detection of cyanide allowing fluorescence and visual identification of the presence of cyanide in the sample (Figure 16). They reported a detection limit of 0.296 µM (17.3 µg/L).

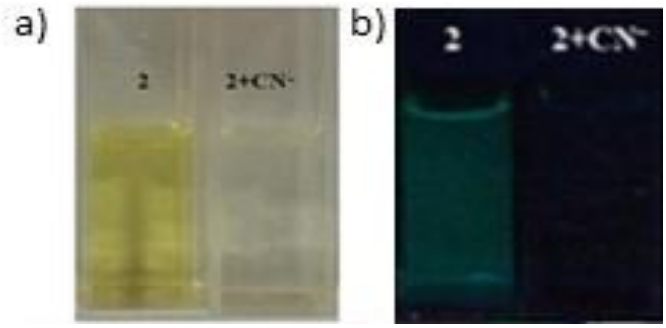


Figure 17. Changes in UV–vis absorption (a) and fluorescence (b) (c, λex=440 nm) spectra of compound 2 in acetonitrile upon the addition of cyanide anions (Wang et al. 2017).

Wang et al. (2017) developed a method using two benzoyl coumarin amide derivatives in which colorimetric changes in presence of cyanide can be observed by the naked eye (Figure 17). However, the samples have to be in acetonitrile. Their limits of detection were 0.016 µM (0.935 µg/L) using absorbance and 0.047 µM (2.75 µg/L) using fluorescence.

Zhen et al. (2017) used triphenylamine-thiophene and dicyanovinyl groups to detect cyanide reporting a detection limit of 0.051 µM (2.98 µg/L) using fluorescence titration (Figure 18). However, the reaction time to get the optimum reaction between the chemosensor and the cyanide (80 min) is lengthy.

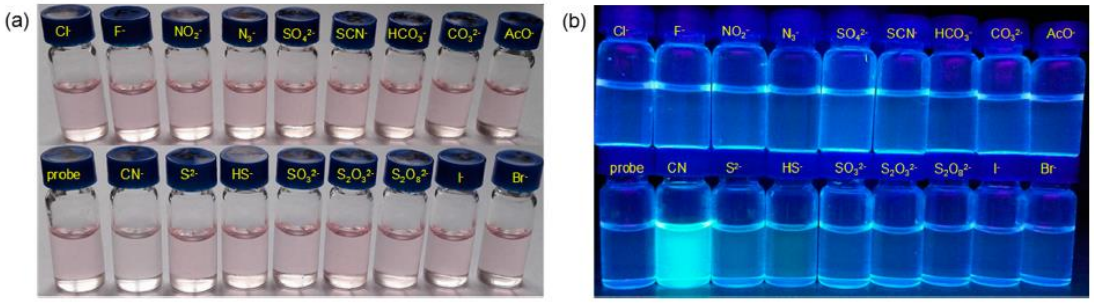


Figure 18. Fluorescence images of triphenylamine-dicyanovinyl probe (5 µM) upon addition of various anions (50 µM [2920 µg/L]) in PBS/DMSO solution under visible light (a) and 365 nm UV lamp (b) (Zheng et al., 2017).

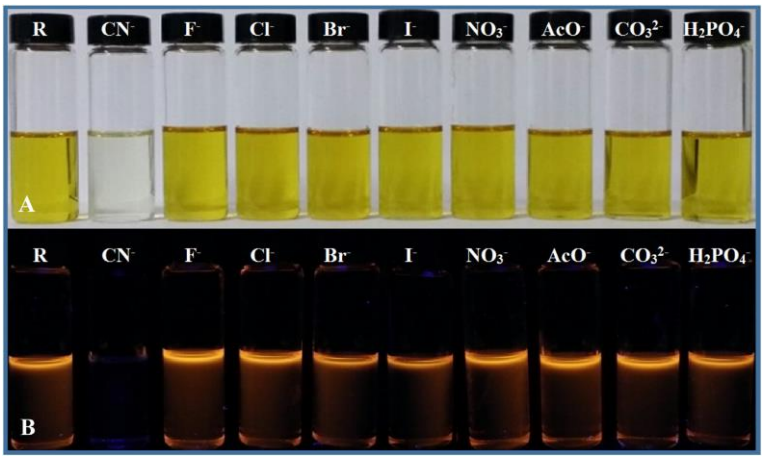


Figure 19. Colour changes observed in aq. HEPES buffer/DMF (80:20 v/v) solution of R [6.25 × 10−5M [3650 µg/L]] upon the addition of various anions; (B) Photograph of R taken under irradiation with UV light after addition of the indicated anion (Kim et al., 2017).

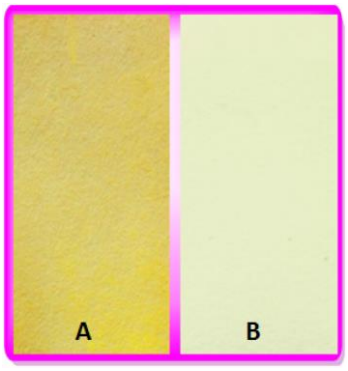


Figure 20. Test strip of R; (B) Color change of the test strip R upon dipping in solution of cyanide ion in water. (Kim et al., 2017).

Kim et al. (2017) studied the use of naphthoquinone-benzothiazole for the detection of cyanide (Figure 19). They also studied the use of strips for practical application immersing them in HEPES buffer and DMF, showing a change in colour when in contact with cyanide (Figure 20). The reported limit of detection was 0.497 µM (29.03 µg/L).

5. Discussion and recommendations

The aim of this desk-based study was to review the analytical methods which are currently available for detecting cyanide and its associated by-products, namely in aqueous matrices including seawater. Many methodologies are available in the scientific literature but most target the analysis of cyanide in blood and plasma matrices and their applicability to live marine fish screening, and therefore analysis in seawater samples requires further study and validation. The methods reviewed can be differentiated into two categories: a laboratory-based; or a field-based technique. For each category, different analytical techniques have been identified as having potential for providing a robust method of detecting cyanide in seawater.

5.2 Laboratory-based techniques

Thiocyanate is thought to be the most suitable target compound for analysis in a laboratory-based test given the length of time elapsed between cyanide exposure and sample analysis. Therefore, an accurate and reliable method for the analysis of SCN^- in the laboratory would provide a baseline for validation measurement against which, other more novel methods of detection, such as newly developed “naked-eye” tests and portable devices can be compared. Once a fit-for-purpose, validated laboratory-based method has been developed it could then be adopted by other laboratories, providing a reference technique for the determination of thiocyanate internationally.

The method of Rong et al. (2005), which uses direct injection of the sample on a HPLC-UV instrument is of particular interest. The main challenge with further development of this technique is that it uses bespoke conditioning of the HPLC column and has the potential for interferences with the UV detector when analysing marine samples. However, it is based on the direct injection of the sample with no sample treatment or preparation and it could potentially be converted into a portable system. The instrumentation, although expensive, is commonly found in water analysis laboratories which would facilitate its implementation on a large scale.

The method presented by Ammazzini et al (2015), which allows the accurate measurement of SCN^- at low concentrations using headspace GC-MS, would also provide a suitable method for development as a laboratory-based analysis for live fish screening. Minimal sample preparation and the use of instruments which are commonly found in water analysis laboratories means that analytical costs for routine operation are expected to be relatively low. The method was developed for the analysis of biological samples and would require

evaluation for seawater samples. However, headspace sampling is particularly appropriate for complex ionic matrices such as seawater. Additionally, the combination of headspace, chromatographic separation and MS allows for a highly selective method. Significantly, MS also allows for the use of isotope dilution which entails the spiking of an isotopically labelled analogue of thiocyanate at the start of the analytical process and which is the recognised gold-standard for accurary. On this basis, the headspace GC-MS method of Ammazzini et al (2015) is recommended for further evaluation over that of the HPLC-UV method of Rong et al (2005).

5.3 Field testing techniques

Screening techniques based on quick and portable methods are ideal for *in-situ* analysis particularly at collection sites, wholesale facilities or at the point of export. Such a system would provide a rapid “yes/no” discrimination of positive and negative samples. Positive samples could then be sent to a laboratory for analysis using the validated reference technique to obtain accurate, measured results using the most precise laboratory-based technique.

Recent developments in methodologies using gold nanoparticles combined with instrumental techniques that are potentially portable can achieve the lowest detection levels for thiocyanate in the ng/L range (Pienpinijtham et al., 2011; Song et al., 2015; Hou et al. 2017). These detection levels are significantly lower to that of levels expected in holding water (based on the limited amount of literature). Particularly, the use of SERS (Raman) (Pienpinijtham et al., 2011; Hou et al. 2017; Dashti, 2016) as a portable devices looks like a promising approach, especially when used with techniques that allow the enhancement of the SCN^- signal. SERS ultimatedly has the advantage of being very specific to the gold thiocyanate complex, which is particularly. While very promising on paper, this approach is still in the domain of R&D, and therefore carry a greater risk of not being fit-for-purpose with significant further development required especially focusing on the need for understanding interferences from marine samples.

RECOMMENDATIONS:

- A laboratory based analytical method is currently the most viable approach for the reliable quantitation of thiocyanate in seawater at concentrations in the lower µg/L.
- The method described by Ammazzini et al. (2015) is recommended for further development as it is promising in terms of simplicity, expected accuracy and detection levels, while having the potential of being routinely applicable in most water

analysis laboratories at relatively low cost. In terms of the timeframe for the development of such a method, it is reasonable to anticipate that a fit-for-purpose method could be produced within six month development exercise, with the caveat that a successful and reliable outcome cannot be guaranteed at this early stage.

- With regards to the development of a portable field test, SERS combined with gold nanoparticles as an enhancer is a particularly promising approach for its extremely high sensitivity and specificity. This approach is currently very much in the R&D domain, and further development of such a method would be expected to take several years. At this early stage, such development is possibly best progressed through a PhD studentship.
- Most of the methods presented in this report have been developed for the analysis of biological samples, therefore the method selected for the analysis of SCN^-, both for laboratory or field-based analysis will need to be optimized to ensure applicability to seawater samples and the levels present in water holding exposed fish. Method development should include, as a minimum, an intra-laboratory validation exercise to meet the requirements of ISO/IEC 17025 (the international standard for the competence of testing laboratories) to characterise method performance criteria such as accuracy, precision, limit of detection, limit of quantitation, specificity and selectivity, linearity and working range in both natural and synthetic seawater. Results will ideally be compared to a technique known to be reliable and accurate which is usually considered to be a validated reference technique for the analysis of SCN^-.
- Ultimately, developed techniques and methods should be validated in different laboratories to guarantee the robustness and accuracy of the results. This will require a “reference” laboratory or proficiency testing scheme provider to coordinate comparative studies to evaluate the robustness of the selected method.

5.4 Toxicokinetics in marine fish

While this report has identified laboratory and field-based methods which are recommended for further development and validation of seawater samples, a lack of understanding of the metabolization products from cyanide in marine fish still exists. Toxicokinetic studies on the process in mammals and in some fresh water fish clearly highlight that the importance and abundance of different metabolites is very much species-dependent. Species-specific studies in target species of marine fish would allow the evaluation of the best alternative target compounds (including study of ATCA, α-KgCN, protein-bound adducts and others) for

the analysis of cyanide exposure. Identification of the most suitable target compound for detecting exposure is likely to vary with time elapsed following the initial exposure event and therefore, different target compounds may be more suitable for laboratory-based testing than for field-based testing.

RECOMMENDATIONS:

- Studies of the toxicokinetics of cyanide in marine fish should be performed using target species based on their value (economic and ecological) and trading importance (e.g. volume traded). Candidate species could include damsels or species from the family of Pomacentridae as key ornamental species and large groupers, such as coral trout, barramundi cod and humphead wrasse for the live food market.

- Further studies into the full suite of cyanide metabolic products (not only targeting SCN^- and ATCA) are needed to understand which metabolites are produced by marine fish and which ones are found at levels that will allow their robust analysis following exposure. Isotopic tracer experiments performed in a laboratory using both natural and artificial seawater, would enable a labelled form of cyanide to be administered to high priority fish species to be tracked through cells and tissues. This would provide information on all the minor pathways and products in marine fish including their retention times, and could potentially identify a more persistent target metabolite.

5.5 Determining the source of cyanide exposure in fish

Further research is also required regarding the measurable levels of cyanide and associated by-products that are detectable following direct exposure to cyanide during cyanide fishing as well endogenous levels found in all biological samples and background environmental levels present in the aquatic environment in collection areas due to natural and anthropogenic pathways. Recent work by Mansfeldt and Hohener (2016) aimed to identify different sources of cyanide (commercial, blast furnace, coal-carbonization) using of isotopic analysis. While they could differentiate commercial from non-commercial cyanide, they could not differentiate between different commercial sources. As a result, the use of isotopic analysis as an approach would not be able to differentiate whether the source of cyanide in fish is due to mining or commercial activities, or from a cyanide fishing source. Research should therefore be focused on establishing the levels of analyte inherently present in a

sample prior to cyanide exposure and specifically, in different collection regions and for different species which are expected to have differing background levels.

The ability to discriminate the presence of cyanide (or by-products) in marine fish from cyanide fishing from other source in the collection region would provide evidence to underpin environmental management of cyanide including the enforcement of waste treatment regulations, pollution events from industry as well as cyanide fishing.

RECOMMENDATIONS:

- Studies on the background levels of cyanide and associated by-products in collection regions are required to provide a baseline against which fish exposed to cyanide fishing can be compared. Water samples and biological samples (fish tissues) should be collected from collection regions around the world and at different times of the year to generate baseline levels of cyanide and by-products.
    - Review existing water quality monitoring programs in collection regions (globally) to assess current cyanide water sampling footprint.
    - Initiate targeted sampling of seawater and fish samples in high-risk (based on industrial activity in area and prevalence of cyanide fishing) collection areas to establish background levels throughout the year.
    - Setting of regional baseline levels (based on the targeted sampling above) against which, live marine fish holding seawater samples can be compared.

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Annex 1: Summary of methods for the analysis of SCN-, ATCA and other metabolites of cyanide.

Target compound	Matrix	Sample preparation	Sample size	Recoveries (in %)	Internal Standard	Analysis	Linearity	Interferences	LODs	Validated	RSD (in %)	Accuracy	Time	Portability	References	Klimisch Score
SCN-	Human serum	Gold-starch surface	N/M	N/M	N/M	Spectroscopy (SERS)	0.05 – 50 µM	CN-	0.05 µM (2.9 µg/L)	N/M	10.5	N/M	N/M	Yes	Pienpinijtham (2011)	**2**
SCN-	Plasma	Dilution and ultrafiltration	~ 20 µL	97 – 104	N/M	IC-UV	1 – 30 mg/L	Not for CN-	500 µg/L	N/M	< 20	N/M	N/M		Saussereau (2007)	**2**
SCN-	Blood	No; use of developed device (MIE)	N/M	31-43	N/M	IC-MS	N/M	N/M	29 µg/L	N/M	N/M	N/M	12 min		Nakamura (2015)	**3**
SCN-	Milk, saliva	Protein precipitation	1 mL	95 - 112	N/M	Spectroscopy (SERS)	1 µg/L – 0.1 mg/L	None knowr	~10 ng/L	Ion chromatography (IC)	< 10	N/M	N/M	Yes	Hou (2017)	**1**
SCN-	Milk, saliva	Milk denaturalized and extracted Saliva centrifuged, filtered and diluted	N/M	100 - 108	N/M	Spectrofluorometer (FRET with fluorescein)	1 – 40 nM	N/M	0.09 nM (5 ng/L)	N/M	0.9 - 8.6	N/M	N/M	Yes	Song^ (2015)	**1**
SCN-	Milk	Milk (liquid and infant formulae) denaturalized and extracted	2 g milk 0.6g infant	91 - 108	N/M	Colorimetric (anti-aggregation of Au nanoparticles)	0.1–1.5 µM	N/M	6.5 nM (0.38 µg/L)	N/M	< 6	N/M	N/M	Yes	Song^ (2016)	**1**
SCN-	Saliva	Centrifuged and dilution	1.5 g	N/M	N/M	ESI- Ion mobility spectrometry	0.01 – 1.00 µg/mL	None for several tested ions	3 µg/L	N/M	3.5	N/M	N/M	IMS yes, coupled to ESI no	Jafari (2010)	**2**
SCN-	Saliva	No	2 µL	96-104	N/M	Colorimetric (paper with Fe III)	0.25 – 20 mM (non-linear)	None from teste ions (F not tested)	0.06 mM (3500 µg/L)	Spectro-photometry	3	N/M	N/M	Yes	Pena-Pereira (2016)	**2**

Target compound	Matrix	Sample preparation	Sample size	Recoveries (in %)	Internal Standard	Analysis	Linearity	Interferences	LODs	Validated	RSD (in %)	Accuracy	Time	Portability	References	Klimisch Score
SCN-	Saliva Serum	SPE nano-sorbent + derivatization	0.5 mL plasma	>94	No	GC-FID	0.6 – 80 ng/mL	N/M	0.2 µg/L	N/M	4.6	N/M	N/M	No	Abdol.-Zadeh (2014)	**2**
SCN-	Water	No	N/M	N/M	N/M	Electrochemical (metalloporphyrins-porphyrins)	N/M	N/M	1-2 ppb (1-2 µg/L)	N/M	N/M	N/M	~10 min		Murphy & Rhyne (2017)	**4**
SCN-	Seawater	Filtration	~ 20 µL	102 - 106	No	HPLC--UV	6.0 µg/L – 2.32 µg/L	N/M	2 µg/L	N/M	3	N/M		Potential	Rong (2005)	**1**
SCN-	Seawater	Pre-concentration	50 mL	N/M	N/M	Spectroscopy (SERS)	N/M	N/M	< 5ppb (<5 µg/L)	N/M	N/M	N/M		Potential	Dashti (2016)	**4**
SCN-	Seawater	Filtration, 4°C	20 µL	N/M	No	LC-Optic fibre	4-400 µg/L	None for iodide and iodate	3 µg/L	HPLC-UV	N/M	0.4%	<6 min	Potential	Silva (2011)*	**1**
SCN-	Synthetic seawater	No	20 µL	N/M	No	LC-Optic fibre	4-400 ug/L	N/M	3.16 µg/L	N/M	N/M	0.4%	<6 min	Potential	Vaz (2012)*	**2**
CN-, SCN- (as CN-)	Waste water, saliva	HS-SPME with Hg	N/M	N/M	N/M	UV-Spectrophotometer	1-50 µmol/L	N/M	0.34 µmol/L CN- (9 µg/L CN-)	Colorimetric method	N/M	N/M	N/M		Al-Saidi (2016)	**2**
CN-, SCN-	Saliva	Alkylative extraction and derivatisation	> 0.5 mL	55-65	N/M	GC-MS	26-2,600 µg/L CN- 290-11,600 µg/L SCN-	N/M	<26 µg/L CN- <290 µg/L SCN-	Colorimetric method	<20	N/M	N/M	No	Paul (2006)	**2**
CN-, SCN-	Plasma	Extraction & derivatisation	N/M	>90	$Na^{13}C^{15}N$. $Na^{13}C^{15}N$	GC-MS (NCI)	10 µM-20 mM CN 500 nM-200 µM SCN- in plasma	Potential for labelled IS	1 µM CN- (26 µg/L CN-) 50 nM SCN- (2.9 µg/L SCN-)	Yes	<9	<15 %	~ 2 h	No	Bhandari~ (2012)	**1**
CN-, SCN-	Blood	Extraction & derivatisation	100 µL		No	GC-MS (NCI)	N/M	N/M	1 µM CN- (26 µg/L CN-) 50 nM SCN- (2.9 µg/L SCN-)	N/M	N/M	N/M	N/M	No	Bhandari~ (2014)	**1**

Target compound	Matrix	Sample preparation	Sample size	Recoveries (in %)	Internal Standard	Analysis	Linearity	Interferences	LODs	Validated	RSD (in %)	Accuracy	Time	Portability	References	Klimisch Score
SCN-	Saliva	Dilution, derivatization	5 µL-	99-103	KS^{13}CN	HS-GC-MS	1–400 µg/g SCN-		<5 ng/g SCN- (<~5µg/L SCN-)	IC-UV-vis	<5	3 %	>1 h	No	Ammazzini (2015)	**2**
ATCA	Blood	Imprinted polymers	N/M	81-89	N/M	HPLC-MSMS	N/M	Histidine, from the tested amino acids	3.5 µg/L	N/M	N/M	>24h	N/M	No	Lulinksi" (2015)	**2**
ATCA	Blood	Precipitation, derivatization & extraction	N/M	86 – 107	ATC-$^{13}C^{15}N_2$	HPLC-MSMS (ESI +)	30 – 900 ng/mL	N/M	0.43 µg/L	N/M	<13	>1h	N/M	No	Giebulotwicz" (2016)	**1**
ATCA	Plasma	MIP-SB	200 µL	Low	ATZA	HPLC-MSMS	N/M	N/M	12 ng/mL	N/M	N/M	N/M	N/M	No	Petrikovics~ (2012)	**3**
ATCA	Urine, plasma	SPE and derivatization	100 µL	84-100%	ATCA-d_2	GC-MS	50-1000 ng/mL	N/M	25 µg/L	N/M	10	N/M	N/M		Logue~ (2005)	**1**
ATCA	Aqueous	Derivatization, extraction	N/M	N/M	N/M	Spectrophotometry		SCN, cysteine	N/M	N/M	N/M	N/M	N/M	No	Basking (2006)	**3**
PB-SCN	Plasma	Precipitation, extraction and derivatization	N/M	N/M	KS$^{13}C^{15}N$	GC-MS	1 – 100 µM	N/M	2.5 µg/L SCN-	N/M	N/M	N/M	> 1h		Youso~ 2010	**2**
PB-SCN	Plasma	Precipitation, extraction and derivatization	N/M	N/M	KS$^{13}C^{15}N$	GC-MS	N/M	N/M	2.5 µg/L SCN-	N/M	N/M	N/M	> 1h		Youso~ 2012	**2**
α-KgCN	Plasma	Precipitated, extracted, filtered	N/M	14-27	α-KgCN-d_4	UPLC-MSMS (ESI -)	0.3-50 µM	N/M	200 nM α-KgCN (37 µg/L)	N/M	<13	N/M	N/M	No	Mitchell~ (2013b)	**3**

Categories: 1🟩: Reliable without restriction; 2🟨: Reliable with restriction; 3🟥: Not reliable, 4🟦: Not assignable (adapted from Klimisch et al., 1997).
Abbreviations: *Target compounds* - SCN: Thiocyanate; CN: Cyanide; ATCA: 2-amino-2-thiazoline-4-carboxylic acid; α-KgCN: α-ketoglutarate cyanohydrin; ESI: Electrospray ionization; FID: Flame ionization detector; FRET: Fluorescence resonance energy transfer; GC: Gas chromatography; HPLC: High performance liquid chromatography; HS: Head space; IC: Ion chromatography; IS: Internal standard; IMS: Ion mobility spectrometry; LC: Liquid chromatography ;LOD: Limit of detection; MIE: Micro ion extractor; MIP-SB: Molecularly imprinted polymer stir bar; MS: Mass spectrometry; MSMS: Tandem mass spectrometry; ND: 2,3-naphthalene dialdehyde; NBA/DNB: p-nitrobenzaldehyde / o-dinitrobenzene; NCI: Negative chemical ionization; N/M: Not mentioned; PB-SCN: protein bound thiocyanate; RSD: Relative standard deviation; SPME: Solid phase microextraction; SERS: Surface enhanced Raman scattering; SPE: Solid phase extraction; THF: Tetrahydrofuran; UPLC: Ultraperformance liquid chromatography; UV: Ultraviolet detector; *: R. Calado group;~: B, Logue group; ": P. Wroczynski group; ^: Song group

Annex 2: Summary of methods for analysis of cyanide. Methodologies that can be applied to aqueous samples and field tests are presented.

Compound	Matrix	Sample prep	Sample size	Analysis	Linearity	Interferences	LODs	Validation	Time	Portable	Reference	Score
CN-	Blood	Mix blood with chemosensor; SPE extraction	0.5 mL	Spectrophotometer (DRUV-vis)(corrin deriv)	0-50 µM	N/M	3 µM (175 µg/L) DRUV And visible	N/M	N/M		Mannel-Croise (2012)	**2**
CN-	Blood	µdiffusion	20-50 µL	Spectrophotometer (cobinamide)	2.2-200 µM/L	S^{2-}	2.2 µM(129 µg/L)	N/M	<4 min	Potential	Tian (2013)	**2**
CN-	Blood	µdiffusion	100 µL	Fluorimeter (NDA-taurine)	3.13-200 µmol/L	None known	0.78 µM(45.6 µg/L)	N/M	N/M	Potential	Jackson (2014)	**2**
CN-	Blood, plasma	Reaction with cobinamide	N/M	Spectrophotometer (cobinamide)	5-100 µmol/L	N/M	0.25 nmol (<58.4 µg/L)	Spectrophotometry	N/M	Potential (strips)	Blackledge (2010)	**2**
CN-	Cassava	Mix with chemosensor and linamarase; SPE	N/M	Colorimetric (corrin derivative)	N/M	N/M	N/M	N/M	N/M		Zelder and Tivana (2015)	**2**
CN-	Aqueous	No	30 µL	Spectrophotometer and visual (spirooxazine)	0.4-500 µmol/L	S^{2-}	0.4 µM(23.4 µg/L)	N/M	N/M	Potential	Zhu (2012)	**2**
CN-	Aqueous	Mix with chemosensor buffered solution	N/M	Colorimetry (Naphtoquinone-indole)	N/M	None for the tested ions	0.01 – 2.1 nM (0.58-123 µg/L)	N/M	N/M		Jayasudha (2017)	**2**
CN-	Aqueous	N/M	N/M	Fluorimeter and colorimetric (tripenylamine-dicyanovinyl)	0-25 µM	None for the tested ions	0.051 µM (2.98 µg/L)	N/M	80 min		Zheng (2017)	**2**

Compound	Matrix	Sample prep	Sample size	Analysis	Linearity	Interferences	LODs	Validation	Time	Portable	Reference	Score
CN-	Aqueous	Mix with buffer	N/M	UV-vis (naphthoquinone-benzothiazole) and visual	N/M	None for the tested ions	0.497 µM (29.03 µg/L)	N/M	N/M	Potential (strips)	Kim (2017)	**2**
CN-		Sample with tetrabutyamonium salts and THF	N/M	Fluorimeter (fluorene-BODYTIP) and visual	N/M	N/M	Fluoresce 0.296 µM (17.3 µg/L)	N/M	N/M		Wang (2015)	**2**
CN-		Sample in ACN	N/M	Fluorimeter, absorption and visible (benzoyl coumarin amide)	N/M	None for the tested ions	0.016 µM (0.935 µg/L) absop; 0.047 nM (2.75 µg/L) Fluorescence	N/M	N/M	Potential (strips)	Wang (2017)	**2**

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