Date: 26th September 2018

To   Jonáš Adam
        Lučební závody Draslovka a.s. Kolín
        Havlíčkova 605, 280 02 Kolín IV
        Czech republic

Re: Ethanedinitrile (cyanogen; CAS 460-19-5)

Dear Adam,

Systox was requested to provide an expert statement on the toxicology of ethanedinitrile (cyanogen; CAS 460-19-5) in the context of setting a TEL.

A. The toxicology of ethanedinitrile (cyanogen), Cyanides and Nitriles

Ethanedinitrile shares a common mode of action as other dissociable cyanides though the presence of the −C≡N functional group. Data on other cyanides in this class should be used in the assessment of ethanedinitrile

Cyanides and Nitriles are characterised by the presence of a −C≡N functional group that is responsible for exerting their characteristic toxicity. Ethanedinitrile (cyanogen or C_2N_2) belongs to the class of cyanides and nitriles that dissociate readily to release CN- ions (Hartung, 1963). Other members of this class are HCN, simple salts (such as sodium, potassium, calcium and ammonium) of HCN and acetone cyanohydrin (Hartung, 1963, ASTDR, 2006[1], ECETOC, 2007). These cyanides are water soluble and under physiological and environmental conditions and will predominantly be present as HCN. In the case of ethanedinitrile, it hydrolyses to HCN, CO_2 and $NH_{3/4}$ (CO_2 and $NH_{3/4}$ being breakdown products of unstable HOCN) with a half-life of 4 minutes (pH 9) and 50 minutes (pH 7) in the dark at 23 °C [2]. The −C≡N functional group of the parent molecule will contribute to the observed toxicity profile as exemplified by the slighter lower toxicity of ethanedinitrile compared with HCN (ethanedinitrile being less readily absorbed than HCN).

Over the last 6 decades, a number of authors have worked on the identification of the molecular mode of action of cyanide poisoning and this was reviewed by ECETOC in 2007. US ATSDR in 2006 described the MOA as follows: "*Cyanide exerts its primary*

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[1] ATSDR included EDN in their review on cyanides (74-90-8; 143-33-9; 151-50-8; 592-01-8; 544-92-3; 506-61-6; **460-19-5**; 506-77-4).

[2] Ajwa, 2015. Effect of pH on hydrolysis of cyanogen in the dark, Ajwa Analytical Laboratories, 1514 Moffett Street, Salinas, California 939 05, ID Study AAL2015-12-A, May 2015

toxicological effects by binding to the metallic cofactor in metalloenzymes, thereby impairing enzyme and cell function. Cytochrome c oxidase (an enzyme in the mitochondrial respiratory chain) is the most significant target of cyanide exposure since its inhibition prevents tissues from using oxygen. The result is a reduction in oxygen sufficient to cause tissue damage (histiotoxic hypoxia) throughout the body, with the most vulnerable tissues being those with high oxygen demands and/or a deficiency in detoxifying enzymes such as rhodanese. The inhibition of oxygen use by cells causes oxygen tensions to rise in peripheral tissues; this results in a decrease in the unloading gradient for oxyhemoglobin. Thus, oxyhemoglobin is carried in the venous blood, which is one biomarker of cyanide exposure. In addition to binding to cytochrome c oxidase, cyanide inhibits catalase, peroxidase, hydroxocobalamin, phosphatase, tyrosinase, ascorbic acid oxidase, xanthine oxidase, and succinic dehydrogenase activities, which may also contribute to the signs of cyanide toxicity".

Since all members of this class share a common MOA then availability of an extensive toxicological database on HCN and cyanides salts (ECETOC, 2007) can be used to supplement the more limited data available on ethanedinitrile.

   B.   **Point of Departure for derivation of the TEL for ethanedinitrile**

The study by Lewis, T.R., et al. (1984) in rats upon which the TEL proposal is made has significant limitations that necessitate other data on analogous chemicals to be taken into account. Available data from reliable studies on the repeated dose toxicity of freely dissociable Cyanides of the same class indicate a NOAEL of at least 10 times higher than that observed in the Lewis study.

EPA staff to derive a tolerable exposure level (TEL) for ethanedinitrile to the general public based upon the NOAEL of 11.2 ppm (23.83 mg/m^3) for decreased weight gain in a 6 month inhalation study of ethanedinitrile in rats (Lewis, T.R., et al. (1984).
As recognised by EPA there is a strong likelihood that the aforementioned study was performed at Bio-test Laboratories (IBT) in the US. IBT was later confirmed of engaging in extensive scientific misconduct which resulted in the indictment of its president and several top executives in 1981 and convictions in 1983. So while there is no immediate reason for disregarding the study there is some concern for the reliability of the data as reported that necessitates independent validation.

The publication that is cited a 13 page scientific paper, including references, and contains both studies in rats and monkey. There were no significant adverse findings in Monkeys and the only adverse finding in male SD rats. The design of the study used 90 rats in total with 6 animals per group per dose level (0, 11 and 25ppm) , referred to as T-C, T-11 and T-25 respectively and groups sacrificed at 2 days, 5 days, 1 month, 3 month and 6 months. On this basis only 6 animals per dose level survived beyond 3 months. The critical effect upon which the NOAEL was established was described as "*The mean*

weight of the T-25 rats completing exposures was 470g which was less than the mean weight of T-11 (589g) and control rats (543g). The overall effect was statistically significant by one-way ANOVA (F=6.51; df=2; 12; P<0.05), and the difference between T-11 and T-C was not (t=1.50, df=9, p=0.17)". Namely, the key finding was a depression in bw gain in 6 animals compared with control that was itself depressed compared with the T-11 group animals. There were no consequential effects in gross pathology, clinical observations, haematology or clinical chemistry in any animals of any species at any dose level.

As recognised by EPA, this test protocol does not compare favourably with the 10 male and 10 female animals per dose level required for the OECD 413, 90 day inhalation study and the "at least 20 animals per sex per group" required for the OECD 452, chronic test guideline.

The study by Lewis, T.R., et al. (1984) in rats is therefore limited by some significant deficiencies that necessitate other data on analogous chemicals to be taken into account. Also considering that ethanedinitrile is recognised as an irritant gas and the fact that irritant gas can depress behaviour and feeding habit in exposed animals, it throws some uncertainty as to whether a depressed b.w. gain in the absence of any adverse effect can be taken significant and form the basis for setting a health based standard.

It is important therefore to look critically at the findings of the study by Lewis, T.R., et al. (1984) in the context of other data on related substances.

As recognised by regulatory agencies across the world soluble cyanides (including ethanedinitrile) are rapidly absorbed via the oral and inhalation routes. The smaller molecules like HCN and fully dissociated salts are also rapidly absorbed via the dermal route. It would therefore be reasonable to expect that, other than for local effects, the findings of repeated-dose studies by oral and inhalation routes would be comparable. In the case of reading across from oral route to inhalation route it is standard practice to assume 100 % absorption for both routes in the absence of chemical specific data. In the case of soluble cyanides including ethanedinitrile this is a reasonable conservative assumption.

Numerous repeated dose toxicity studies on cyanides have been conducted by the oral and inhalation route. Some of the oral studies were designed to evaluate the impact of cassava diets on animal health and included groups that received cyanide supplemented food diets for comparison of the effects. ECETOC in their review in 2007 identified several key repeat dose toxicity studies that they considered were well conducted and of high reliability, namely, three 90-day guideline studies in rats and mice in drinking water (NaCN - Hébert (NTP), 1993) and in rats by inhalation (ACH - Monsanto, 1984), and the 1-year study in rats in diet (KCN - Philbrick et al, 1979). The available NOAELs from these studies are broadly consistent: 25.6 mg CN^-/kg bw/d in mice (NaCN - Hébert

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(NTP), 1993), 12.5 mg CN⁻/kg bw/d in rats (excluding reproductive effects) (NaCN - Hébert (NTP), 1993) or approximately 10.4 mg CN⁻/kg bw/d (ACH - Monsanto, 1984) in rats. ECETOC noted that, consistent with the recognized MOA of cyanides "*these doses are approximating the point on the steep dose-response curve at which acute lethality might be expected as suggested by the signs of acute toxicity and mortality seen in the ACH study by Monsanto (1981) 28-day study at 59.9 ppm (212 mg/m³ or 65 mg CN⁻/m³, equivalent to 8.5 mg CN⁻/kg bw/d)*".

Based upon a mol. wt. of 26 for CN⁻ and 52 for ethanedinitrile, these would be equivalent to 51.2 (mice/NaCN; Hébert (NTP), 1993), 25 (rats/NaCN; Hébert (NTP), 1993) or approximately 20.8 (rats/ACH; Monsanto, 1984) mg C_2N_2/kg bw/d all of which are considerably higher than the 2.05 mg C_2N_2/kg bw/day that EPA derived from the study in rats of Lewis et al. (1984).

These NOAELs calculated for ethanedinitrile from well conducted, reliable studies would suggest that the decreased weight gain observed in 6 rats in the 6 month inhalation study of ethanedinitrile (Lewis et al., 1984) is not of toxicological significance.

C. **Data that agencies have considered key when setting health based standards for cyanides**

A reference concentration for chronic inhalation exposure (RfC) for HCN was set by the US IRIS programme in 1994 using the study of El Ghawabi et al., 1975. This study is unreliable and unsuitable for use in setting a health-based standard.

HCN was reviewed under the US IRIS programme in 1994. A reference concentration for chronic inhalation exposure (RfC) for HCN was estimated, based upon CNS symptoms and thyroid effects observed in the occupational study reported by El Ghawabi et al (1975) and the claim by the author that occupational exposure to between 4.2 and 12.4 ppm cyanide is linked with development of goitre and a range of subjective symptoms). ECETOC reviewed the study of El Ghawabi in 2007. They summarised the weaknesses of the study as follows:

> "*At first glance, the study of El Ghawabi et al (1975) suggests that these clinical effects are consistent with occupational exposures of between 4.2 and 12.4 ppm. The problem with accepting this interpretation is that the levels of urinary thiocyanate reported were too low to have caused the development of goitre (larger studies in humans refer: Cliff et al, 1986 in Section 9.2.2) and there were no effects on thyroid hormones. Indeed, the urinary thiocyanate levels reported were more consistent with levels of occupational exposure of 1 mg/m³ (0.9 ppm, 8-h TWA). This brings into question the exposure data presented (36, 15 minute breathing zone samples) (Section 5.2.2). Because no shift average (8-h TWA) monitoring data were collected it could be that these data, although perhaps taken during specific tasks, were not representative of the accumulative exposure*

across a full shift (unlike the urinary thiocyanate data). If current exposures were as low as the thiocyanate data indicates the question remains what caused the goitre and the subjective symptoms that were reported. Goitre is not an acute response and is therefore indicative of a more prolonged exposure to cyanide. Indeed, the absence of effects on thyroid hormones would suggest that the thyroid disturbance that caused the goitre had been some time prior to the current study. Similarly, the questionnaire is likely not to have distinguished between current and past symptoms so it is quite possible that past exposures might have been much higher and these had caused both the goitre and the subjective symptoms. Because of this uncertainty, it must be concluded that the study of El Ghawabi et al (1975) is not sufficiently reliable to be used as the basis for establishing an occupational NOAEL".

The numerous inconsistencies within this study and between this and other studies in the same industries (also reviewed by ECETOC in 2007) suggest that this study is not reliable and should not be used to set a health-based standard for cyanides.

D. **Considerations for selecting Uncertainty Factors to compensate for uncertainty when extrapolating from data in animals to humans.**

EPA applied an overall Uncertainty Factor of 100 to the modified point of departure. This one error and one area for possible improvement. Firstly, the Interspecies AF for toxicokinetics is essentially allometry differences between rats and humans and this was already accounted for when different respiratory rates and body weights were taken into account in the modification of the NOAEL in rats. Secondly, EPA did not adequately consider chemical specific information on the known MOA and metabolism of cyanides when assigning AFs. These considerations justify use of a lower overall AF.

The EPA methodology for deriving a TEL is described as consisting of 2 stages. Firstly, they convert an inhalation concentration given in rats to a systemic dose on a mg/kg basis then convert this to a human systemic dose on a mg/kg basis. Secondly, they then apply a 100-fold assessment factor to this to address uncertainties in the inter- and intra-species extrapolation and finally convert this to an inhalation concentration in humans.

The 100-fold assessment factor is described by EPA as consisting of the following components:
1) An AF of 4 for Interspecies (laboratory animal to human) uncertainties

2) An AF of 5 for Intraspecies variation (differences and sensitivities within the human population)

3) An AF of 1 for Exposure Duration (extrapolation from a subacute or subchronic exposure to a chronic exposure)

4) An AF of 2 for Database Quality (Completeness and consistency of the data, reliability of alternative data, e.g. read across)
5) An AF of 3 for Modifying Factor (Professional assessment of the scientific uncertainties of the key study)

EPA recognised that AFs were not necessary for route to route extrapolation and extrapolation from a LOAEL to NOAEL. This gave an overall AF of 120 that was rounded down to 100.

While it should be acknowledged that assignment of AFs is in part science policy, there are aspects of this process that are based in science fact. The process of applying AFs has been developed by the international risk assessment community over the past 60 years and has found its way into regulatory guidelines and practices across the world.

The AFs for interspecies differences is comprised of 2 components, toxicokinetics (TK) and toxicodynamics (TD). The TK/TD split was originally proposed based on underlying data in rodents and humans related to basic physiological parameters, cardiac output, renal and liver blood flows (major determinants of clearance/ elimination) and are consistent with an approximately four-fold difference (according to the three-quarter power of the ratio of the body weight between rats and humans).

As recognised by EPA, typically a default AF of 100 (10x10) is applied where there is no knowledge of the MOA and toxicokinetics/dynamics of the substance in animals and humans. Neither is the case with Cyanogen. Hence deviation from using default conservative AFs is justified (WHO/IPCS 1994, 2005; EFSA, 2006; Meek et al. 1999, 2002; US EPA, 1994 and 2011).

In the case of ethanedinitrile there is a sound basis for deviation from use default AFs. Ethanedinitrile is structurally similar to cyanide and other nitriles. Although definitive metabolism and disposition data for cyanogen in humans or animals are not available, it is known that ethanedinitrile is converted in the body to yield one mole of hydrogen cyanide and one mole of cyanate (McNerney and Schrenk, 1960; Flury and Zernik, 1931, as cited in Kopras, 2012). Clinical signs noted in cyanogen-exposed animals are comparable to those noted in hydrogen cyanide-exposed animals. The MOA of ethanedinitrile, like other cyanides, is via interruption of cellular respiration by inhibiting cytochrome oxidase, thus blocking electron transfer to oxygen (Rieders, 1971). It's MOA and mechanism of detoxification is highly conserved within animal species. Data on HCN and cyanide salts is particularly relevant to the assessment of ethanedinitrile and has a significant impact on the selection of the most appropriate AFs.

The impact of correctly sub dividing the TK and TK component factors together with the informed use chemical specific information on the MOA etc. impacts the AFs used by EPA in the derivation of a TEL for ethanedinitrile in the following way:

1)   Interspecies (laboratory animal to human) uncertainties
         EPA: 4

The AF for interspecies uncertainties is conventionally comprised of Toxicokinetic (TK) and Toxicodynamic (TD) subfactors.

The Toxicokinetic (TK) subfactor

The TK factor principally accounts for allometric differences between rats and humans i.e. already accounted for when EPA modified to POD to take account of differences in respiratory rate and body weight between rats and humans. This double counting introduces a factor of 4 unnecessarily so if the modification is done at the POD the AF for TK differences should be 1.

In way of illustration:
Starting point: Male rats repeatedly exposed to cyanogen at 25 ppm for 6 h/day, 5 days/week for up to 6 months, experienced only decreased body weight. NOAEL 11.2 ppm (23.83 mg/m^3)

Accounting for differences in respiratory rate and body weight between rats and humans occurs in two part of the EPA calculation highlighted below:
"1) Rat atmospheric exposure NOAEL converted to an average daily intake (mg/kg bw dose) of ethanedinitrile. This was completed to adjust for rodent respiratory intake versus human respiratory physiological parameters. = [Rat alveolar ventilation rate (L/min)] x (min/hr) x [rat exposure time (hrs/day)] x (ethanedinitrile concentration mg/m^3) x (m^3/1000 L) / rat BW (kg) = 0.117 L/min x 60 min/hr x 6 hrs/day x 23.83 mg/m^3 x 1 m^3/1000 L / 0.35 Kg = 2.87 mg/kg bw/day x (5 of 7 day exposure correction) = 2.05 mg ethanedinitrile/kg bw day"
2.)"TEL = NOAEL / 100 = 0.0205 mg ethanedinitrile/kg bw/day TEL mg/kg bw/day dose converted to an atmospheric exposure level for humans ethanedinitrile mg/m^3 = TEL (mg/kg bw/day) x human bw (kg) / daily human ventilation rate (m^3/day) = [0.0205 mg/kg bw/day x 70 kg bw] / 20 m^3/day = 0.072 mg/m^3 (0.034 ppm)."

In this way the TEL derivation converts the NOAEL in rats to an equivalent NOAEL in humans taking into account differences in breathing rates and body weights in rats and humans. While correcting for ventilation rate and body weight differences between rats and humans is necessary, this also is accounted for within the TK factor for allometric differences between rats and humans (4 in the case of rats to humans)

So by subsequently applying an interspecies AF of 4 takes account of allometric differences twice. This double counting introduces a factor of 4 unnecessarily so if the modification is done at the POD the AF for TK differences at this stage should be 1.

For further reading and validation EPA is referred to ECH TGD Chapter R.8: Characterisation of dose [concentration]-response for human health, page 26, Table R. 8-4 - when to apply allometric scaling (AS) factor.

If EPA were to delete the correction for ventilation rate and body weight differences between rats and humans from the calculation then as recognised by EPA a factor of 4, the default value used in the REACH technical guidance document and by ECETOC and WHO/IPCS to account for TK and metabolic differences between species, would apply.

The Toxicodynamic (TD) subfactor

Toxicodynamics refers to the molecular, biochemical, and physiological effects of toxicants or their metabolites in biological systems and are result of the interaction of the biologically effective dose of the ultimate (active) form of the toxicant with a molecular target within the cell.

Although EPA makes reference to concerns over TD the application of an overall AF of 4 for Interspecies differences infers the use of a subfactor of 1.

EPA States "*In general, the main TD concern of chronic cyanide exposure in humans is disruption of thyroid metabolism from its detoxification to thiocyanate. Importantly, this effect appears to not have been manifested in the Lewis study. However, the EPA is concerned with the fact that different forms of cyanides (organic and inorganic) also appear to be capable of inducing different types of toxicities (e.g., kidney and CNS effects) with less known modes of action; and that there are different NOAELs in studies of similar duration from different cyanide forms. Thus different types of cyanides seem to manifest in different types of toxicity when exposed chronically. It is also unknown as to what the etiological basis was for the significant weight loss effect observed in the Lewis study rats and what it may correlate to in humans.*"

This level of concern deserves some detailed inspection and comment.
   a) "*In general, the main TD concern of chronic cyanide exposure in humans is disruption of thyroid metabolism from its detoxification to thiocyanate.*" Indeed, the most sensitive adverse effect observed in humans chronically exposed to inorganic and organic cyanides is their effect on thyroid function and hormones. ECETOC made an exhaustive review of the available data on such in both humans and concluded that "*Other studies (nutritional, epidemiological and clinical) indicate the absence of thiocyanate-mediated toxicity to the thyroid gland from daily doses equivalent to an occupational exposure (8-hour) of 7.5 mg CN^-/m^3 (time-weighted average) for humans with sufficient dietary iodine and normal kidney function. The acutely toxic cyanide concentration that can be tolerated by humans may be of the same order of magnitude. Sensitive sub-*

populations would include individuals with insufficient dietary iodine, insufficient thiosulphate supply (e.g. in the case of malnutrition) and impaired renal function." This Human NAOEL equates to an occupational exposure (8-hour) of 5 mg C_2N_2/m^3 or 2.5ppm for the general population (7.5x8/24x2(mol wt C_2N_2/HCN)). The proposed TEL is sufficiently protective of this concern.

b) *"Importantly, this effect appears to not have been manifested in the Lewis study."* As indicated the Lewis study may suffer from some limitations. Most importantly is the fact that effects on thyroid and thyroid hormones are well recognised and the NOELs in the fully valid studies on other cyanides cited by ECETOC (51.2 (mice/NaCN; Hébert (NTP), 1993), 25 (rats/NaCN; Hébert (NTP), 1993) or approximately 20.8 (rats/ACH; Monsanto, 1984) mg C_2N_2/kg bw/d) are significantly higher than the highest concentration used in the Lewis study (equivalent to 2.87 mg C_2N_2/kg bw day – based upon EPA's calculation minus the 5/7 day correction). On this basis it is no surprise that effects on thyroid hormones or organ weight were not detected.

c) "*However, the EPA is concerned with the fact that different forms of cyanides (organic and inorganic) also appear to be capable of inducing different types of toxicities (e.g., kidney and CNS effects) with less known modes of action; and that there are different NOAELs in studies of similar duration from different cyanide forms. Thus different types of cyanides seem to manifest in different types of toxicity when exposed chronically.*" This not true. The ECETOC review of 2007 went to considerable lengths to explain the challenges with designing studies with cyanides due to the steep dose response curve for acute toxicity(lethality), the proximity of this to the dose response curve for repeated exposure effects and the observation that dosing regimens employed in less well designed studies fail to account for bolus toxicity often resulting in acute poisoning. This is further confounded by practical aspects such as tainting of drinking water by cyanides (bitter almonds taste) resulting in sustained abstinence and gorging; requirement of adequate dietary iodide and fluctuating background hormone levels. In those studies referred to by EPA as having "*different types of toxicities (e.g., kidney and CNS effects)*" these are without exception due to the contribution of acute toxicity and/or bad study design and interpretation and not *"less known modes of action"* which do not occur. Indeed, ECETOC also reviewed the clinical experience of use of sodium nitroprusside used to treat hypertensive emergencies and to improve the hemodynamics of chronic heart failure and acute myocardial infarction for many years, noting that "*From studies in human patients, infusion of up to 100 to 150 µg sodium nitroprusside/min (44 ̶ 66 µg CN⁻/min) did not lead to any signs of cyanide toxicity (Schulz et al, 1982; Schulz, 1984). These authors claimed that chronic exposure above these levels will cause fatal poisoning.*" This provides direct evidence in humans that chronic exposure at sub-lethal doses of cyanides does lead to "*different types of toxicities*".

d) *"It is also unknown as to what the etiological basis was for the significant weight loss effect observed in the Lewis study rats and what it may correlate to in*

humans." Here I have to agree with EPA other than it was a claimed "significant reduced body weight gain" not loss. There is no logical basis for this effect in the absence of any effect on any other parameter. I suspect that this is an artifact of the small group sizes used – only 6 animals in the T-25 test group at 6 months forming the POD. The lower terminal body weights of the control (T-C) rats compared with the T-11 animals points to a wide variability in animal weights. Since no individual animal weights or interim kill animal weights were included in the paper it is impossible to determine if this effect was substance related or not.

In conclusion, therefore, the analysis of concern for TD variability is not supported by the available information when reviewed properly.

Rather, in the case of ethanedinitrile, once within the body the toxic entity is HCN and differences in the MOA of HCN between rats and humans, or indeed any mammalian species, are not expected. Cyanides are commonly recognised (Hartung, ECETOC and many reviewers) as amongst the most rapidly acting chemical toxicants known. Their MOA is highly conserved amongst living species from plant to bacteria, insects, animals and birds (see ECETOC review of 2007 for a comprehensive review) rendering it unlikely that there will be significant TD differences between rats and humans. Accordingly a TD factor of 2.5 conventionally applied for remaining differences is not required.

In this respect the chemical specific information supports an AF of 1 for Toxicodynamic differences as inferred by EPA's assessment.

2.)   Intraspecies variation (differences and sensitivities within the human population)
EPA: 5

Here EPA states "*Although the default UF is 10 (unless TK and TD factors are considered) the EPA utilized a factor of 5 as defined by ECETOC as insufficient information is available to quantitatively estimate the variability in human susceptibility to chronic ethanedinitrile exposure and the TK and TD of ethanedinitrile in humans is also unknown.*"

EPA goes on to state "*The primary concern of chronic cyanide toxicity is associated with the cyanide detoxification metabolite thiocyanate. The effect of the thiocyanate metabolite on human thyroid function triggers a wide range of concerns in fetuses, children, and adults with the possibility of gender sensitive differences (see discussion of susceptible populations in the US-EPA IRIS report). The staff believe a default value of 5*

would be sufficient to account for human population variation and the unknown potential effects of ethanedinitrile to humans."

On this basis there are two substances of toxicological concern to EPA that require addressing i.e. the toxic moiety containing the functional $-C\equiv N$ group (whether ethanedinitrile or HCN) and the detoxification metabolite, thiocyanate.

EPA recognises the contribution of TK and TD components in determining the intraspecies AF but as they consider that there are not quantifiable leans towards the use of a default AF of 5 recommended by ECETOC without further justification. By using the default AF of 5 recommended by ECETOC over a value of 10 which more conventionally used, EPA assumedly recognise that intraspecies (within human) variability is likely to be low.

As recognised by EPA the AF for intraspecies differences within the general population and used by agencies across the world is 10. Again this is comprised of 2 components and it is necessary to look at the component factors to understand how chemical specific information may inform on the overall AF. The TK/TD splits for interspecies differences have been assigned as being 3.16-fold (normally rounded to 3.2) in the absence of data allowing them to be determined more precisely.

EPA claims that there is no quantifiable data to allow them to be determined more precisely. This is true there is no quantifiable data but EPA have assigned other AFs within this assessment based upon qualitative assessments so it is valid to include a closer consideration of the available qualitative data.

The Toxicokinetic (TK) subfactor

a) The toxic moiety containing the functional$-C\equiv N$ group (whether ethanedinitrile or HCN)

Ethanedinitrile is a relatively small (Mol wt. 52), water soluble molecule that is likely to be absorbed readily by the inhalation routes of exposure.

As stated above, ethanedinitrile is converted in the body to yield one mole of hydrogen cyanide and one mole of cyanate (McNerney and Schrenk 1960; Flury and Zernik, 1931, as cited in Kopras, 2012). Half-life data do not exist for ethanedinitrile metabolism although based upon the observation that the acute toxicity is about a factor of 2.5 less than that of hydrogen cyanide it is likely that some combination of hydrolysis and metabolic conversion to HCN e.g. via P450 enzymes may play a role in its systemic toxicity (Silver et al., 1999).

The detoxification of cyanides in humans is the same as in rodents and is well characterised (again refer to the ECETOC review for detailed literature). The main route of metabolism of HCN and other nitriles is enzymatic (rhodanese) trans-sulphuration into thiocyanate. Inter-individual variation in serum rhodanese activity can vary by a factor of 6 (Nawata et al, 1991) or 3 to 8 (Narendranathan et al, 1989). However, rhodanese is present in all body tissues in considerable excess and not rate-limiting (Himwich and Saunders, 1948; Schulz et al, 1982), unlike thiosulphate, which may be only available in the body in small amounts depending on the nutritional status (Schulz et al, 1982). No major polymorphisms have been identified to date. A rare hereditary disease, Leber's optic atrophy has been linked by some authors to a deficiency in rhodanese activity (Cagianut et al, 1984; Wilson, 1965, 1983; Poole and Kind, 1986), but this was not confirmed by other authors (Pallini et al, 1987; Berninger et al, 1989; Whitehouse et al, 1989). Protein deficient populations are more susceptible to cyanide intoxication as thioamino acid levels are reduced. Other sensitive sub-populations will include people with impaired renal function and low dietary iodide. On this basis the default AF for toxicokinetics of 3.2 is appropriate.

On this basis the default factor of 3.2 for Toxicokinetics differences appears justified.

  b) the detoxification metabolite, thiocyanate

In this regard it is worth pointing out that there is reliable data on the effects of thiocyanate after prolonged exposure. In a 2-year study, F344 rats (20/sex/group) were given sodium thiocyanate (0.32% NaSCN) in drinking water for 5 days per week for up to 112 weeks. The dose levels were approximately 250 mg NaSCN/kg bw/d as stated by the authors (corresponding to 179 mg SCN^-/kg bw/d). No increase in mortality and no increase in tumour incidence were reported compared to controls. No other effects were reported in the publication (Lijinsky and Kovatch, 1989). This NOAEL of 2.2 mM SCN^-/kg bw/day is 40 times higher than the highest concentration of 0.055 mM C_2N_2/kg bw/day used in the Lewis study clearly indicating that thiocyanate toxicity is not of concern.

Furthermore, former use of sodium or potassium thiocyanate as an antihypertensive drug and experiences with antihypertensive therapy with sodium nitroprusside suggests that serum levels of 20 to 40 µg SCN^-/ml would not lead to adverse effects in healthy humans although they may aggravate pre-existing goiter (reviewed by ECETOC, 2007).

Consequently, any concern over the toxicity of thiocyanate would be more than adequately addressed by the use of the default factor of 3.2 for Toxicokinetics difference.

The Toxicodynamic (TD) subfactor

As stated earlier, toxicodynamics refers to the molecular, biochemical, and physiological effects of toxicants or their metabolites in biological systems and are result of the interaction of the biologically effective dose of the ultimate (active) form of the toxicant with a molecular target within the cell. By definition it excludes factors that determine TK.

Again, EPA's concern relate to both the toxic moiety containing the functional−C≡N group (whether ethanedinitrile or HCN) and the detoxification metabolite, thiocyanate.

a) The toxic moiety containing the functional−C≡N group (whether ethanedinitrile or HCN)

Clinical signs noted in ethanedinitrile-exposed animals are comparable to those noted in hydrogen cyanide-exposed animals consistent with the MOA being like other cyanides, i.e. via interruption of cellular respiration by inhibiting cytochrome oxidase, thus blocking electron transfer to oxygen (Rieders, 1971). Consideration of the structure of ethanedinitrile (e.g. using OECD toolbox) does not alert to the presence of other functional groups other than the −C≡N group so it is unlikely that there are other AOPs that require consideration regarding acute toxicity. Since, this recognised MOA and is highly conserved within animal species so not only is there anticipated to be very limited variation in TD between individuals but also data on HCN and other nitriles that share the same common MOA will be relevant to the assessment of ethanedinitrile. So, in regard to direct toxicity, the use of an overall AF of 1 for TD differences would appear appropriate in regard to the functional−C≡N group.

b) the detoxification metabolite, thiocyanate

Thiocyanate is a potential thyroid disruptor due to its capacity to inhibit the uptake of iodide by the thyroid. Thiocyanate also interacts with the enzymatic reactions associated with iodide organification and thyroid hormone synthesis. There is no information in the literature to indicate that there are inter-individual differences in sensitivity.

EPA's concern over "*a wide range of concerns in fetuses, children, and adults with the possibility of gender sensitive differences (see discussion of susceptible populations in the US-EPA IRIS report)*" relates to the MOA of thiocyanate on the thyroid and thyroid hormones and the effects thereof. Such concerns are only valid if there are perturbations in hormone levels outside the normal homeostatic range. However, in the absence of any effect on the thyroid and hormone levels it is hard to entertain how these consequential adverse effects might occur. As stated previously the NOAEL for effects on thyroid hormones is estimated to be 5 mg C_2N_2/m^3 or 2.5ppm for the general population which is much higher than any proposed TEL for ethanedinitrile. Accordingly no additional AF is considered necessary.

3.) **Route to route extrapolation (difference in route animals were exposed versus humans)**
EPA: NA

No route to route extrapolation is required as both the study route in rats and the target rout in humans is inhalation.

4.) **LOAEL to NOAEL (extrapolation from a LOAEL when a NOAEL is not available)**
EPA: NA

The Point of Departure based upon the study or Lewis or those of Hébert (NTP) and or Monsanto are all NOELs or NOAELs so no adjustment is required.

5.) **Exposure Duration (extrapolation from a subacute or subchronic exposure to a chronic exposure)**
EPA: 1

EPA state that "*the key study used to calculate the TEL was an inhalation study of six months in duration. Chronic toxicity studies typically consist of an exposure duration of 12 months or more, thus, the Lewis study is considered to be sub-chronic in nature. Although most risk assessment guidelines state the need for an UF of 2 to be added for calculating risk from a sub-chronic to a chronic study, it is believed to be based on the fact that most sub-chronic studies are of 90 days in exposure. The California OEHHA indicates an UF of 1 can be used for studies that have a duration that is >12% of an animal's life span. Staff note that the lifespan of a laboratory rat is circa 2 years so 12% of that is ~3 months.*"

This is consistent with standard practice. Furthermore, the study of Lewis is of sufficient duration based upon the premise that the known MOA would suggest that that even by 90 days a steady state conditions would have been achieved and that conduct of a longer duration study is unlikely to change the NOAEL. An overall AF for the study duration of 1 is therefore considered appropriate.

6.) **Database Quality (Completeness and consistency of the data, reliability of alternative data, e.g. read across)**
EPA: 2

EPA states "*Although there is a very large and extensive data base on systemic cyanide toxicity that can be used in the qualitative assessment of the systemic toxicity potential of ethanedinitrile gas there is only one repeated dose toxicity study available to quantitatively assess its hazard potential. Furthermore, it is important to point out that*

the vast majority of the studies assessing cyanide toxicity were conducted using oral (water or dietary) exposures. Thus, the data base available to assess other toxicity endpoints is reliant on read-across oral studies as opposed to inhalation. The utilization of these data in the absence of TK information on ethanedinitrile is a concern that needs to be accounted for."

There are two points that are worth addressing:

a) "*Although there is a very large and extensive data base on systemic cyanide toxicity that can be used in the qualitative assessment of the systemic toxicity potential of ethanedinitrile gas there is only one repeated dose toxicity study available to quantitatively assess its hazard potential*".
The first part of the sentence is correct there is a very large and extensive database on the systemic toxicity of "cyanides". The ECETOC review of 2007 bears witness to this. However this statement is linked to the claim that "*there is only one repeated dose toxicity study available to quantitatively assess its hazard potential*". This is untrue if one extends the scope of the determination to include other chemicals of the class of cyanides and nitriles that dissociate readily to release CN^- ions (Hartung, 1963). Other members of this class are HCN, simple salts (such as sodium, potassium, calcium and ammonium) of HCN and acetone cyanohydrin (Hartung, 1963, ASTDR, 2006, ECETOC, 2007). If these chemicals are included then since there are other reliable oral and inhalation studies available on these chemicals (see ECETOC, 2007) this claim is misleading.

b) "*Furthermore, it is important to point out that the vast majority of the studies assessing cyanide toxicity were conducted using oral (water or dietary) exposures. Thus, the data base available to assess other toxicity endpoints is reliant on read-across oral studies as opposed to inhalation. The utilization of these data in the absence of TK information on ethanedinitrile is a concern that needs to be accounted for.*"
Again, the first part of the sentence is correct there is a very large and extensive database consisting of studies employing oral administration (gavage, dietary, drinking water) on the systemic toxicity of "cyanides". Again, the ECETOC review of 2007 bears witness to this. For repeat dose toxicity of ethanedinitrile apart from the limited study of Lewis there is a reliance on read-across oral studies (2 studies by Hébert (NTP)) but there is also an inhalation study on acetone cyanohydrin (ACH) (Monsanto 1984). EPA states that "*The utilization of these data in the absence of TK information on ethanedinitrile is a concern that needs to be accounted for.*" This concern does not take into account the fact that the ADME of these read across salts of HCN and ACH is well understood and indicate rapid absorption and wide distribution of the common toxic moiety, HCN, within the body so they represent a worst case from which to read-across to ethanedinitrile.

Hence, although chemical specific information is lacking on ADME of ethanedinitrile read across to these chemicals would suggest that the conservative default AFs for intraspecies variation in humans would be sufficient to compensate for these uncertainties. An AF of 1 therefore considered appropriate.

7.) Modifying Factor (Professional assessment of the scientific uncertainties of the key study)
EPA: 3

EPA assigns a modifying factor of 3 to account "*professional assessment of the scientific uncertainties of the key study*". This type of factor is referred to as "Quality of whole database" in ECHA TGD terminology. ECHA TGD on page 30 describes this factor as "*To account for deficiencies in the available data set and in identifying its magnitude, the assessor should consider both the data lacking and the data available. When there are deficiencies in the toxicity studies considered crucial to provide useful information for establishing the starting point, extra caution should be taken to address this scientific uncertainty in deriving the DNEL. Further, in order to account for data gaps and deficiencies in the available data set and in identifying its magnitude, the assessor should consider the nature of the effect occurring in particular organ systems, endpoints as well as at different life stages.*"

ECHA TGD goes on in the same paragraph to state *"Special consideration should also be given to alternative data, e.g. in vitro data, (Q)SAR, read across or chemical categories."*

In this regard, EPA's concern over the reliability of the key study and in particular its recognition that the study was likely conducted in the notorious Industrial Bio-test Laboratories (IBT) is fully justified. EPA should also perhaps have noted that reduced body weight gain was not just limited to the 25ppm group compared with controls but was also seen in the control group compared with the 11ppm group and each of these findings was only based upon a group of 6 animals. Since there was an absence of any corresponding effect in any parameter measured questions the toxicological relevance of the finding.

EPA explains that "*Part of the UF assigned is also associated with study duration*" for which it assigned an AF of 1.

It is contested that in line with ECHA TDG TGD guidance and recognised practice use of read-across data could both cast some light on the reliability of the Lewis study findings as well as increase overall confidence in the assessment by introducing other high quality, reliable studies upon which a more robust POD could be established. Again, consistent

with general practice any uncertainty in the read-across of data should be addressed under the respective subfactors under the interspecies AF. In this regard the established MOA/metabolism etc. of cyanides are a significant consideration.

## 8.)	Options for TEL derivation

In a perfect world there would be a guideline study with ethanedinitrile or even a study in humans that is of high reliability and precision from which we could set a TEL. As is typically the case this is not the situation that we find ourselves in. Reality is that we have limited options.

a)	Firstly, we can set a TEL based upon a "far from ideal" study (Lewis) and account for the uncertainties that are associated with this study with additional modifying factors. The disadvantage of this approach is that the POD in the Lewis study is considerably lower than we would expect when we compare the findings with other reliable studies with other chemicals that chare the same MOA etc. The resulting TEL is extremely low and inconsistent with human experience with other cyanides and nitriles. For the intended purpose of this TEL this may have adverse socioeconomic or feasibility impacts. This is effectively the TEL currently proposed by EPA.

b)	Another option is that we can set the TEL on the findings from robust studies on other cyanides that share the common MOA using a process of read-across. This is not without some uncertainty so we would have to address this aspect, for example, under the AF for interspecies extrapolation. This approach relies on the level of confidence that the assessor has on the read-across rationale i.e. hypothesised MOA and whether uncertainties introduced by the read across process have been adequately addressed.

c)	Yet another option is what may be considered as a midway position, i.e. that of setting the TEL based upon the "far from ideal" study (Lewis) and use informed or chemical specific AFs (CSAF) to minimise the distorting effect of the lack of confidence in the POD.

Whichever approach is taken it is perhaps essential to identify clearly the contribution of available chemical specific information to both TK and TD components of the intraspecies and interspecies AFs thereby ensuring transparency and consistency and optimising confidence in any deviation from default assumptions.

TEL based upon option b)

This approach would perhaps take the lowest NOAEL determined in reliable repeated dose toxicity studies on cyanides. ECETOC in their review in 2007 identified several key repeat dose toxicity studies that they considered were well conducted and of high reliability, namely, three 90-day guideline studies in rats and mice in drinking water (NaCN –Hébert (NTP), 1993) and in rats by inhalation (ACH - Monsanto, 1984), and the 1-year study in rats in diet (KCN - Philbrick et al, 1979). Within the precision of such studies the available NOAELs are broadly consistent when represented as cyanide ion i.e. 25.6 mg CN⁻/kg bw/d in mice (NaCN – Hébert (NTP), 1993), 12.5 mg CN⁻/kg bw/d in rats (excluding reproductive effects) (NaCN - Hébert (NTP), 1993) or approximately 10.4 mg CN⁻/kg bw/d (ACH - Monsanto, 1984) in rats.

While most studies use the oral route with only one being by inhalation, and most being 90-day and only one 1-year duration, the known MOA of cyanides and nitriles with the extensive evidence in humans from exposure to HCN, cyanogens and therapeutic nitroprusside would indicate that other than the effects on thyroid hormones there are no "other adverse effects of concern". In the case of effects on the thyroid hormones these are evident well within the time period of the available studies so no AF for study duration need be applied.

Using a weight-of-evidence approach these studies performed independently by different investigators, in different laboratories and at different times collectively they form a robust data set upon which to set a TEL for cyanides and ethanedinitrile. Based upon a mol. Assuming 100% conversion of ethanedinitrile to the more toxic HCN, and taking into account the molecular weights of 26 for CN- and 52 for C_2N_2, the reported NOAELs in these studies are equivalent levels of. 51.2 (mice/NaCN; Hébert (NTP), 1993), 25 (rats/NaCN; Hébert (NTP), 1993) or approximately 20.8 (rats/ACH; Monsanto, 1984) mg C_2N_2/kg bw/d.

Taking the lowest NOAEL of approximately 20 mg C_2N_2/kg bw/day and applying a total AF of 12.8 ($4_{InterTK}$ x $1_{IinterTD}$ x $3.2_{IntraTK}$ x $1_{IntraTD}$ x $1_{Duration}$ x$1_{Database\ quality}$ x $1_{modifying}$) this gives a human NOAEL of 1.56 mg C_2N_2/kg bw/day. Correcting this for human body weight (70 kg bw) and respiratory volume (20 m³/day) = 5.46 mg/m³ (2.53ppm)."

This does not include any additional modifying factors for uncertainty in the use of read-across as this is determined by policy and professional judgement. As a long time serving member of the Scientific Committee of ECETOC and the co-chairperson responsible for the 2007 review on Cyanides I have high confidence in scientific basis of the read-across hypothesis and the inherent conservatism of assuming that the POD based upon studies in cyanide salts and ACH apply to ethanedinitrile. A tolerable exposure level (TEL) for ethanedinitrile to the general public could be based upon the NOAEL of 11.2 ppm (23.83 mg/m³) for decreased weight gain in a 6 month inhalation study of ethanedinitrile in rats (Lewis, T.R., et al. 1984).

TEL based upon option c)

This option derives the TEL based upon the NOAEL of 11.2 ppm (23.83 mg/m^3) for decreased weight gain in a 6 month inhalation study of ethanedinitrile in rats (Lewis, T.R., et al. 1984) and uses informed or chemical specific AFs (CSAF) to minimise the distorting effect of the lack of confidence in the POD.

By applying an overall chemical specific AF (CSAF) of 12.8 (4 $_{InterTK}$ x 1$_{IinterTD}$ x 3.2 $_{IntraTK}$ x 1 $_{IntraTD}$ x 1 $_{Duration}$ x1$_{Database\ quality}$ x 1 $_{modifying}$) to the adjusted POD of 4.255 mg ethanedinitrile/m^3 based upon the NOAEL of 11.2 ppm (23.83 mg/m^3) for decreased weight gain in a 6 month inhalation study of ethanedinitrile in rats (Lewis, T.R., et al. (1984) results in a TEL of 0.33 mg ethanedinitrile/m^3 for humans (0.153 ppm).

9.)   Conclusions

The draft documentation for derivation of a TEL for ethanedinitrile has serious flaws in the way it deals with uncertainty regarding the key study in male rats by Lewis (1984) and incorporates this into a total AF of 100 to account for uncertainty in extrapolating from studies in animals to humans. The resultant TEL is extremely low and inconsistent with human experience with other cyanides and nitriles. For the intended purpose of this TEL this may have adverse socioeconomic or feasibility impacts.

One aspect that is highlighted for further consideration is the current incorrect application of an intraspecies AF for toxicokinetics that should not be applied when allometry has already been used to adjust the inhalation POD. This is contrary to regulatory guidelines and general practice.

In terms of the key study by Lewis (1984) and the toxicological adverse effect identified, that of reduced body weight gain at 25ppm in male rats, this was in the absence of any other signs of systemic toxicity and inconsistent with other more robust studies with other cyanides that share the same mode of action as ethanedinitrile strongly pointing to it not being of toxicological significance. EPA recognises weaknesses in this study and raising other uncertainties that cannot be confirmed or quantified but compensate for this by applying a somewhat arbitrary, additional modifying factor thereby driving down the resultant TEL as opposed to reading across to reliable data on other dissociable cyanides like HCN, salts of HCN and ACH which would allow the use of chemical specific information to deviate from default assumptions. This preferred way of dealing with uncertainty by looking for alternate studies upon which to depart is a practice recognised and recommended by WHO IPCs and regulatory agencies across the world.

Due to the weaknesses in the available data on ethanedinitrile and the complexity of the available data on cyanides and nitriles different options are available upon which to set a TEL for ethanedinitrile. All of these available options are associated with some degree of uncertainty. The TEL could be based upon the weak study of Lewis but in this case use of chemical specific AFs over arbitrary or default AFs is recommended to ensure transparency and avoid still driving down further the already conservative POD. Alternatively, a weight of evidence approach based upon the findings of several, high quality studies on cyanides that share the same MOA as ethanedinitrile i.e. via CN^- could be used, but this may be judged as also being associated with some uncertainty regarding the read-across hypothesis that is equally challenging to quantify.

Whichever approach is finally taken by EPA it is recommended that the correct use of adjustments allometric differences between rats and humans and appropriate chemical specific AFs are used.

On behalf of Systox Limited

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