Clarification on the identity of the test substance and on resulting NOEC

"Murrell, H.R. and Leak, T. (1995): Chronic Toxicity of Hydrogen Cyanamide to Daphnia magna under Flow-Through Test Conditions; ABC Laboratories, Columbia (Missouri), Final Report #41942"

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I. Introduction

A study on chronic toxicity of a substance to the aquatic invertebrate *Daphnia magna* is performed in order to gain information about the aquatic risk of this substance. For this reason, it is essential that the study report defines clearly and unambiguously to which substance and to which purity of the substance the test concentrations and consequently the derived effect values refer.

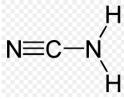
The study documentation "Chronic Toxicity of Hydrogen Cyanamide to Daphnia magna under Flow-Through Test Conditions" which is subject to this position paper comprises the study report (121 pp.) and a raw data package (331 pp.). The study report is composed of the main study report (p. 1-33) and three annexes containing amongst others statistical and analytical data and a copy of the study protocol.

II. Clarification on test substance

II.1. Test substance

The test materials as mentioned in the study report were (summary, p. 9):

> Hydrogen cyanamide solution; received 9-22-94, (Batch #424801), The purity listed as 50% w/w; clear liquid. ^{14}C Hydrogen cyanamide (Lot# 950530), received 5-31-95; specific activity = 55 mCi/mmole.

"Hydrogen cyanamide" is a synonym for "cyanamide" and both terms refer to the chemical substance with the structural formula  and a molecular weight of 42.04 g/mole.

DORMEX®, another term mentioned in the study, is the trade name of the 50% w/w aqueous solution of pure (hydrogen) cyanamide which was employed as test substance.

The test was performed using ^{14}C radiolabelled pure (hydrogen) cyanamide as indicator substance and was isotopically diluted with DORMEX®. From this isotopically diluted ^{14}C cyanamide solution the test solutions (= dosing solutions) were prepared.

Five test concentrations (plus a water control) are specified in the study report (summary, p. 9):

Nominal Test Concentrations:	Control, 0.025, 0.050, 0.10, 0.20, and 0.40 mg/L
Mean Measured Test Concentrations:	0.023, 0.049, 0.10, 0.21, and 0.41 mg/L

Unfortunately, the study report (p. 1-33) gives no clear advice as to which substance these values and also the test results refer, to DORMEX® or to pure (hydrogen) cyanamide. The following terms regarding the test material were used in the study report: Hydrogen cyanamide, Hydrogen cyanamide solution, a.i. [active ingredient], DORMEX®, Hydrogen cyanamide (DORMEX®) technical standard.

For this reason, the study results (study report, summary, p. 9) do not permit to draw unambiguous conclusions with respect to the ecotoxicological impact of cyanamide on the aquatic invertebrate species *Daphnia magna*.

Results: 21-Day EC_{50} = >0.41 mg/L
21-Day NOEC = 0.21 mg/L
21-Day LOEC = 0.41 mg/L
21-Day MATC = 0.29 mg/L

MATC: = Maximum Acceptable Toxicant Concentration

A lot of other statements in the study report are also ambiguous and can easily lead to the conclusion that the 50% w/w cyanamide solution is meant. For example, see the citation below (study report, abstract, p. 10, 1st paragraph):

"A flow-through chronic toxicity study was conducted with *Daphnia magna* exposed to […] five concentrations of Hydrogen cyanamide, (Lot No. 424801, CAS 420-04-2, at 50% w/w purity) […] Mean measured concentrations of Hydrogen cyanamide, determined by LSC, were 0.023, 0.049, 0.10, 0.21, and 0.41 mg/L."

The intention of this position paper is to show that the mentioned test concentrations and **the determined study results refer to pure (hydrogen) cyanamide** and not to the test material DORMEX®, a 50% (w/w) aqueous solution of pure (hydrogen) cyanamide.

This fact can most precisely be demonstrated following the descriptions of the preparation of the dosing solution (= stock solution):

II.2. Preparation of the stock solution

The preparation of the stock solution is described in the study report on page 13:

The Hydrogen cyanamide (Dormex®) technical standard (batch #424801) was received from SKW Trostberg on September 22, 1994, in good condition and was assigned ABC Reference #TS-7688. This sample was observed to be a clear liquid and was stored refrigerated. Sample purity was given to be 50% Hydrogen cyanamide, 50% water, on a weight to weight basis. This sample was used to prepare preliminary test solutions and to isotopically dilute the radiolabeled standard.

The ^{14}C-cyanamide sample (lot #950530) was received from American Radiolabeled Chemicals on May 31, 1995, in good condition and was assigned ABC Reference #RS-8305. This sample was observed to be a clear liquid (in ethanol) and was stored refrigerated. Specific activity was given as 55 mCi/mmole, and total activity as 1 mCi. Radiochemical purity was given to be 99%. This sample was isotopically diluted with the technical standard (TS-7688) to prepare dosing solutions for the definitive test.

The ^{14}C-cyanamide standard (RS-8305) was quantitatively transferred with ABC reagent water to a volume of 25 mL. The resultant solution was designated SS-8305. LSC analysis performed on replicate aliquots of SS-8305 confirmed its concentration at 31.5 μg/mL. This was stored in a refrigerator.

The dosing solution for this study was prepared in the following manner: A total of 810 mg nonradiolabeled Hydrogen cyanamide active ingredient (TS-7688) was mixed with 110 μg of ^{14}C-cyanamide (3.5 mL of SS-8305) in a total volume of 2 L ABC reagent water. The calculated concentration of this solution was 405 mg/L. The calculated specific activity of this solution was 395 dpm/μg.

One of the main reasons for the uncertainty concerning the interpretation of test concentrations and test results might be due to the misleading wording in the previous paragraph: "A total of 810 mg non-radiolabelled Hydrogen cyanamide active ingredient (TS-7688) […]". "Hydrogen cyanamide active ingredient" would refer to pure (hydrogen) cyanamide whereas TS-7688 specifies DORMEX® which contains 50% w/w pure (hydrogen) cyanamide (see above). Consequently, this phrase is contradictorily. In order to solve the problem, the raw data had to be consulted.

That the 810 mg non-radiolabelled "Hydrogen cyanamide active ingredient" actually refer **to pure (hydrogen) cyanamide** and **not to Dormex** is clearly demonstrated by calculations presented in the raw data, appendix III (Figure 1). To achieve this 810 mg pure (hydrogen) cyanamide in the stock solution 1620 mg DORMEX® have to be applied.

Taking into account this information provided by the raw data, the preparation of the stock may be described as presented in the following example:

> The ^{14}C-cyanamide standard* (laboratory reference no. RS-8305) was quantitatively transferred with water to a volume of 25 ml (= laboratory reference no. SS-8305). The concentration of this aqueous ^{14}C-cyanamide solution was measured by liquid scintillation counting (LSC). The concentration of ^{14}C-cyanamide was determined to be 31.5 µg/mL.
>
> 3.5 mL of aqueous ^{14}C-cyanamide solution containing 110 µg of ^{14}C-cyanamide were mixed with 1620 mg of DORMEX® and made up with ABC reagent water to 2000 mL. The resulting stock solution had a nominal concentration of 405 µg pure (hydrogen) cyanamide/mL. This corresponds to a specific radioactivity of 395 dpm/µg pure (hydrogen) cyanamide
>
> (*^{14}C-cyanamide standard: total activity 1 mCi, specific activity 55mCi/mmol, radioactive purity 99% (study report, p. 13))

The above mentioned values can be calculated as follows:

> 110 µg of ^{14}C-cyanamide ≙ 0.00262 mmole ^{14}C-cyanamide ≙ 0.1441 mCi** ≙ 3.2×10^8 dpm***
>
> 110 µg of ^{14}C-cyanamide ≙ 3.2×10^8 dpm ≙ 1620 mg of DORMEX® ≙ 810 mg pure (hydrogen) cyanamide ≙ 810000 µg pure (hydrogen) cyanamide
>
> 810000 µg pure (hydrogen) cyanamide ≙ 3.2×10^8 dpm
>
> 1 µg pure (hydrogen) cyanamide ≙ 395 dpm
>
> (** 1 Ci = 3.7×10^{10} dps = 2.22×10^{12} dpm; *** dps: decays per second; dpm: decays per minute)

ABC LABORATORIES, INC.

OBSERVATIONS AND/OR REMARKS FORM

Test Material: ^{14}C - Cyanamide Protocol No. EPA 5-73-1-B

Study Director: H. Murrell Study #: 41942

6/7/95

Conc. of SS-8305 $\frac{9.14 \times 10^3 dpm \times 1 ml}{0.01 ml \times 0.1 ml} = 9.14 \times 10^7 dpm/ml$ (from LSC: AAS 41942:01 6/8/95)

$$\frac{9.14 \times 10^3 dpm \times 10 ml}{0.01 ml \times 0.1 ml} \Big/ \left[\frac{55 mci}{mmol} \times \frac{1 mmol}{42.04 mg} \times \frac{2.22 \times 10^9 dpm}{1 mci}\right]$$

$= 0.0315$ mg/ml

Volume of Hcf material needed: 0.110 mg / 0.0315 mg/ml = 3.49 ml

1.62 g Hydrogen Cyanamide (TS-7688, 50%) were weighed and placed in a 2-L Volumetric flask. (Balance 1626-141 6/9/95)

3.50 ml SS-8305 were placed in same flask. Filled the flask to mark w/ ABC reagent water. labeled as SS-8335

Conc. of SS-8335: 1.62 g × 50% = 0.81 g = 8.10×10^5 ug = 810 mg

810 mg / 2.0 L = 405 mg/L

Specific Activity: $\frac{3.50 ml \times 9.14 \times 10^7 dpm/ml}{8.10 \times 10^5 ug + 110 ug} = 395$ dpm/ug

NOTE: Individual entries must be dated and initialed.

Reviewed by: Donald A Rothdruff Date: 1-12-1995

Study Director: H. Murrell Date: 9-23-95

FORM # 55 (11/25/91) (FDI)

ABC 41942R PG 00137

Figure 1: Preparing of the stock solution

(p. ABC 41942**R** PG 000137 of the raw data, appendix III)

II.3. Test solutions

The test solutions (5 concentrations plus control) were prepared from the stock solution (also called diluter solution or dosing solution) using an automatic diluter system. This diluter system was calibrated before test start.

In order to measure the test concentrations during the test, samples were taken at 9 particular times and the radioactivity was counted. After test finalisation the mean measured concentrations were determined.

The following table (Figure 2) provides the nominal as well as the mean measured test concentrations. They are identical to those which were presented in the study summary (please see above, p. 2).

It could be clearly shown in the explanations above that the diluter/stock solution refers to pure (hydrogen) cyanamide and not to the 50% aqueous solution DORMEX®. Hence, it is unquestionable that the test concentrations/test levels provided in the table below also relate to pure (hydrogen) cyanamide.

TABLE II

Measured Concentrations of ^{14}C-Hydrogen Cyanamide Equivalents During the 21-Day Chronic Toxicity Study with *Daphnia magna*

		Measured Concentration (mg/L)					
Sample	Control	Level 1 (0.025)[a]	Level 2 (0.050)[a]	Level 3 (0.10)[a]	Level 4 (0.20)[a]	Level 5 (0.40)[a]	Diluter Stock (400)[a]
Day -5[b]	<MQL[c]	0.0222 0.0234	0.0476 0.0474	0.102 0.103	0.197 0.208	0.400 0.398	393
Day 0	<MQL[c]	0.0240 0.0240	0.0455 0.0481	0.103 0.108	0.202 0.201	0.405 0.406	402
Day 4	<MQL[c]	0.0243 0.0243	0.0485 0.0507	0.101 0.102	0.201 0.208	0.404 0.407	392
Day 7	<MQL[c]	0.0204 0.0204	0.0468 0.0476	0.0998 0.0985	0.195 0.198	0.390 0.402	403
Day 11	<MQL[c]	0.0277 0.0261	0.0569 0.0567	0.123 0.122	0.241 0.249	0.473 0.469	---
Day 12	<MQL[c]	0.0230 0.0228	0.0472 0.0489	0.102 0.101	0.201 0.205	0.397 0.404	---
Day 13	<MQL[c]	0.0238 0.0232	0.0475 0.0467	0.102 0.101	0.197 0.206	0.392 0.394	---
Day 14	<MQL[c]	0.0231 0.0221	0.0465 0.0464	0.104 0.106	0.197 0.204	0.403 0.407	396
Day 21	<MQL[c]	0.0225 0.0222	0.0461 0.0488	0.0988 0.102	0.198 0.197	0.403 0.403	393
Mean[d]	---	0.023	0.049	0.10	0.21	0.41	400
SD	---	0.0018	0.0034	0.007	0.016	0.024	5
CV, %	---	7.8	6.9	7.0	7.6	5.9	1.3
Recovery, %	---	92	98	100	105	103	100

[a] Nominal concentration(mg/L).
[b] Pre-exposure concentrations, with the exception of diluter stocks, are not included in the summary statistics.
[c] MQL = Minimum Quantifiable Limit; mean MQL for the study = 0.0052 mg/L.
[d] Value rounded to 2 significant figures.

Please note: The nominal/calculated concentration of the stock (= diluter/dosing) solution was 405 mg/L pure (hydrogen) cyanamide; please refer to p. 4 and Figure 1. However, in the table above (Figure 2) and in most parts of the raw data the nominal/calculated concentration of the stock (= diluter/dosing) solution was given as 400 mg/L pure (hydrogen) cyanamide; presumably in order to simplify the calculation of the five nominal test concentrations. Nevertheless, this procedure did not influence the test results since they refer to measured concentrations.

Figure 2: Overview on test concentration measured during the test

(Study report, p. 24)

Additional proof for the fact that the concentrations of the diluter/stock solution and the test solutions, refer to pure (hydrogen) cyanamide can be found in the study protocol.

ABC PROTOCOL NO. FIFRA E-72-4-B Page 4

***Note:** Written confirmation of percent purity along with specific activity and molecular weight, where applicable, must be provided.*

Purity Correction (Please check the appropriate statement.)

__X__ Test concentrations will be based on purity.

_____ Test concentrations will be based on total product.

Test Design (Please check the appropriate statement.)

__X__ Flow-Through – Daphnids/Chamber: ___10___

_____ Static Renewal – Daphnids/Chamber: _____

Analytical Confirmation (Please check the appropriate statement.)

__X__ Analytical confirmation will be performed by ABC Laboratories, Inc.

_____ Sponsor requests no analytical confirmation.

_____ Analytical samples will be collected and shipped to sponsor for analysis.

Special Instructions and/or Comments ☒ Check here if not applicable.

Figure 3: Test specific information
(Study report, Annex I, p. PG 00038)

The phrase "Test concentrations will be based on purity" considers the pure (hydrogen) cyanamide content in the test substance DORMEX®. The purity and thus the pure (hydrogen) cyanamide content of DORMEX® is 50% w/w (see above, p. 2).

II.4. Clarified test concentrations

It could be shown that the **test concentrations** as mentioned in the study report refer to **pure (hydrogen) cyanamide**. They were:

- Nominal: 0.025, 0.050, 0.10, 0.20, and 0.40 mg pure (hydrogen) cyanamide/L
- Measured: 0.023, 0.049, 0.10, 0.21, and 0.41 mg pure (hydrogen) cyanamide/L
  Measured*: 0.023, 0.049, 0.104, 0.206, and 0.409 mg pure (hydrogen) cyanamide/L

The test concentrations corresponding to the 50% w/w aqueous solution of pure (hydrogen) cyanamide (= DORMEX®) would be:

- Nominal: 0.050, 0.10, 0.20, 0.40, and 0.80 mg DORMEX®/L
- Measured: 0.046, 0.098, 0.20, 0.42, and 0.82 mg DORMEX®/L
- Measured*: 0.046, 0.098, 0.208, 0.412, and 0.818 mg DORMEX®/L

* These values are expressed in more detail and derived from the measured single values as provided in Fig. 2, p.6.

III. Clarification on test results

Preparing the draft CAR the competent authority checked the raw data and identified a mistake in transferring the raw data into the calculation sheets:

The number of “total alive young” in replicate C of control group at test is not 1205 as stated in the statistical part of the report but 1505.

ABC LABORATORIES, INC. - ENVIRONMENTAL BIOLOGY DIVISION
SAS PROGRAM RUN DATE OF 08AUG95 USING DATA FILE B:\41942.ARD
21-DAY CHRONIC TOXICITY STUDY WITH DAPHNIA MAGNA EXPOSED TO DORMEX
ABC STUDY NUMBER: 41942
COMPARISON TO CONTROL GROUP FOR YOUNG PER ADULT REPRODUCTIVE DAY (YAD)
PRINTOUT OF RAW DATA

TREATMENT	REPLICATE	REPROD. DAYS	INSTAR1	INSTAR2	INSTAR3	INSTAR4
CONTROL	A	150	134	194	192	488
CONTROL	B	150	104	30	378	497
CONTROL	C	150	76	38	~~47~~ 347	391
CONTROL	D	150	124	0	428	418

TREATMENT	INSTAR5	INSTAR6	INSTAR7	TOTAL INSTAR	YAD
CONTROL	409	316	182	1915	12.77
CONTROL	378	80	267	1734	11.56
CONTROL	366	0	287	~~1205~~ 1505	~~8.03~~ 10.03
CONTROL	371	86	218	1645	10.97

Figure 4: Results for control
(Study report, Annex I, p. PG 00091)

That implies in sum a higher “mean total alive young” for the control as result from 4 replicates related to reproduction days; it must be 11.33 instead of 10.83.

	Mean Measured Concentrations	Mean % Survival	Mean YAD
	Control	100.0	~~10.83~~ 11.33
	0.023 mg/L	100.0	10.91
	0.049 mg/L	97.5	11.50
NOEC	0.10 mg/L	95.0	10.49
LOEC	0.21 mg/L	100.0	9.64 *
	0.41 mg/L	97.5	8.54

* statistically significant

Figure 5: Summary of data and statistical analyses
(Study report, Table VI, p. 28)

A recalculation of LOEC/NOEC based on the new value for the control was carried out with Dunnett and Williams test.

The resulting **NOEC** is:

0.104 mg pure (hydrogen) cyanamide/L, corresponding to **0.208** mg DORMEX®/L

IV. Conclusion

Based on the fact that the test concentrations refer to pure (hydrogen) cyanamide and considering the recalculated LOEC and NOEC values, the determined effect values are:

- **21-Day NOEC 0.104 mg pure (hydrogen) cyanamide/L**
- 21-Day LOEC 0.21 mg pure (hydrogen) cyanamide/L

This corresponds to:

- **21-Day NOEC 0.208 mg DORMEX®/L**
- 21-Day LOEC 0.416 mg DORMEX®/L