National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

FRONT OFFICE FOOD AND PRODUCT SAFETY

Evaluation of the derivation of the Point of Departures for the risk assessment of the cyanotoxins Cylindrospermopsin and Microcystin-LR

Risk assessment requested by:	Office for Risk Assessment & Research (BuRO)
Risk assessment performed by:	RIVM
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Nederlandse samenvatting

In periodes van warm weer kan in oppervlaktewater bloei optreden van cyanobacteriën. Deze bacteriën kunnen toxische stoffen produceren (cyanotoxines) die in het water terecht kunnen komen. Het gebruik van dit verontreinigde water voor het besproeien van landbouwgewassen of het drenken van vee kan schadelijk zijn voor mensen en dieren. Deze beoordeling gaat over de giftigheid van twee van zulke cyanotoxines: cylindrospermopsine (CS) en microcystine-LR (MCLR). MCLR is giftig voor de lever en remt proteïnefosfatase. CS remt de eiwitsynthese en is giftig voor een aantal organen /weefsels: lever, milt en longen; het beïnvloedt de spermiogenese en het wordt verdacht van genotoxiciteit.

Het Franse onderzoeksinstituut ANSES heeft in januari 2019 twee opinies gepubliceerd over de cyanotoxines MCLR en CS. ANSES heeft voor beide toxines gezondheidskundige grenswaarden afgeleid op basis van twee, in de literatuur gerapporteerde, toxiciteitsstudies. Voor beide studies had ANSES allereerst een BMDL afgeleid maar oordeelde dat deze niet gebruikt konden worden als uitgangspunt (‘point of departure’) voor een gezondheidskundige grenswaarde omdat deze BMDLs niet aan de door ANSES gehanteerde criteria voldeden.
Bureau Risicobeoordeling en Onderzoek (BuRO) heeft het Front Office Voedsel- en Productveiligheid (FO) gevraagd om op basis van beide toxiciteitsstudies de volgende vragen te beantwoorden:

1. Kan met behulp van Benchmark Dose modellering een point of departure worden afgeleid voor de studie van Chen et al. (2011) en Chernoff et al. (2018)?
2. Welke point of departure leidt het FO af op basis van deze studies?

Het FO is er in de beantwoording van deze vragen vanuit gegaan dat deze twee studies de kritische studies zijn voor deze twee stoffen en heeft geen literatuuronderzoek uitgevoerd naar deze stoffen. Het FO heeft onderzocht of met behulp van benchmark dose modellering een point of departure kan worden afgeleid voor CS en MCLR. Daarbij heeft het FO ook de aanpak van ANSES beoordeeld. RIVM komt tot de conclusie dat benchmark dose modellering van de gegevens gerapporteerd door Chen et al. (2011) en Chernoff et al. (2018) geschikt is voor het afleiden van een BMDL. Voor MCLR is de laagste BMDL 0,13 µg/L (gerelateerd aan morfologische afwijkingen in spermacellen) en

voor CS is de laagste BMDL 9,4 µg/kg lg (gerelateerd aan het relatieve levergewicht). Onder voorbehoud dat de studies van Chen et al. (2011) en Chernoff et al. (2018) voor MCLR en CS als key-studies kunnen worden beschouwd, kunnen deze BMDLs worden gebruikt als point of departure voor een risicobeoordeling.
Tot slot heeft RIVM (puntsgewijs) commentaar geleverd op de aanpak van ANSES, met als belangrijkste opmerking dat het verwerpen van de BMDL en het terugvallen op een NOAEL/LOAEL[1] onjuist is.

Subject
In January 2019, the French Agency for Food, Environmental and Occupational Health & Safety (ANSES; l'Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail) has released two opinions on the cyanotoxins microcystin-LR (MCLR; CAS nr. 101043-37-2) and cylindrospermopsin (CS; CAS No. 143545-90-8). For the derivation of Health Based Guidance Values (HBGVs) for both toxins, ANSES made use of two (key) toxicity studies published in the scientific literature. Based on the published dose-response relationships, ANSES performed a Bench Mark Dose (BMD) analysis for MCLR and CS. For both toxins ANSES reported that the calculated BMD lower confidence limits (BMDLs) could not be used for the derivation of an HBGV because they did not meet the criteria applied by ANSES. BuRO would like the Front Office for Food and Product Safety (FO) to verify the findings by ANSES and, if possible, derive BMDLs based on the two respective key studies.
BuRO would like to carry out a risk assessment for the occurrence of cyanobacteria in surface waters and publish an advice on permissible levels of cyanotoxins in these waters. Surface waters can be used for the sprinkling of food crops and for drenching of farm animals. Presence of cyanobacteria could pose a health risk for consumers and animals. An important starting point for this risk assessment is the derivation of the Points of Departure (PoDs) for the toxins mentioned.

Questions
FO is requested to address the following questions, on the basis of the two key studies as selected by ANSES.

1. Can, by application of Dose-Response modelling, Points of Departure (PoDs) be derived from the studies by Chen et al (2011) and Chernoff et al (2018)?
2. Which PoDs can be derived for microcystin-LR (MCLR) and cylindrospermopsin (CS)?

Answers
1) From the data on MCLR reported by Chen et al. (2011), as well as the data on CS reported by Chernoff et al. (2018) PoDs can be derived.

2) For MCLR the BMDL was 0.13 µg/L (related to 'sperm head abnormalities'). For CS the BMDL was 9.4 µg/kg bw (related to 'relative liver weight'). Provided the studies by Chen and Chernoff are the key toxicity studies for these two cyanotoxins, the BMDL values derived from these studies are suitable PoDs for toxicological risk assessments.

Introduction
During warm periods, bloom of cyanobacteria (blue-green algae) can be observed in fresh surface waters. Cyanobacteria can produce toxic substances (cyanotoxins) which can end up in the water. Use of contaminated water for spraying crops or drenching of farm animals could be harmful to humans and livestock.
This evaluation addresses the toxicity of two of such cyanotoxins: microcystin-LR (MCLR) and cylindrospermopsin (CS). MCLR is a liver toxin and inhibits protein phosphatase. CS

[1] NOAEL: No Observed Adverse Effect Level; LOAEL: Lowest observed Adverse Effect Level.

inhibits protein synthesis and is toxic to a variety of tissues/organs: kidneys, spleen and lungs, it may affect spermiogenesis and is suspected of genotoxicity. The latter may affect the derivation of a Health-Based Guidance Value (HBGV) for CS.
In January 2019, ANSES published two risk assessment reports, one on MCLR and one on CS (ANSES 2019a, b). ANSES derived for each of these toxins a health based guidance value (HBGV). The Tolerable Daily Intake (TDI) for MCLR amounted to 1 ng/kg bw/day and for CS a TDI of 140 ng/kg bw/day was calculated. The TDI for MCLR was based on a No Observed Adverse Effect Level (NOAEL) taken from a study by Chen et al. (2011) in which adverse effects on spermiogenesis in mice were observed after 3 or 6 months of exposure. The TDI for CS was derived from a Lowest Observed Adverse Effect Level (LOAEL) derived from adverse liver effects observed in a 90-day oral toxicity study in mice, published by Chernoff et al. (2018). ANSES also performed a Bench Mark Dose (BMD) analysis for MCLR and CS but reported that the calculated BMD lower confidence limits (BMDLs) could not be used for the derivation of an HBGV because they did not meet the criteria applied by ANSES.

By request of the Office for Risk Assessment & Research (BuRO) the Front Office Food and Product Safety (abbreviated as FO) has evaluated the BMD analyses of ANSES and their derivation of the PoDs. Subsequently, FO performed new BMD analyses for both cyanotoxins. Starting point for the assessment by the FO has been that the studies by Chen et al. (2011) and Chernoff et al. (2018) are the critical studies for the derivation of a PoD for these two cyanotoxins.

Benchmark Dose (BMD) analysis of MCLR
The key study that ANSES used for the derivation of the PoD of MCLR is by Chen et al. (2011). Chen et al. studied the effects of chronic low-dose exposure to microcystin-LR on sperm quality and testicular function in male mice. MCLR was orally administered to male mice via drinking water at concentrations of 0, 1, 3.2 and 10 µg/L for 3 and 6 months. It was a study that focussed on only a few specific endpoints. ANSES focused on two endpoints, namely: number of spermatozoids and sperm mobility. They considered the default value of 5% for the Benchmark Response (BMR), as recommended by EFSA in their guidance on BMD (EFSA, 2017a), too low. ANSES derived a BMR based on the BMR 1 SD, an approach that is regularly used by US EPA (US EPA, 2012). In this approach, a change in the mean response that is equal to the standard deviation (SD) in the control animals is considered as the BMR. In this way, ANSES arrived at a BMR of 14.5% for number of spermatozoids and 17.5% for sperm mobility. For their dose-response modelling, ANSES made use of the EFSA webtool (EFSA, 2017b), which at that time was based on PROAST 65.6, in which model averaging was not yet implemented. The current version of the EFSA webtool is an implementation of PROAST version 67.0, which does include model averaging.

ANSES calculated the following Benchmark dose lower confidence limits (BMDLs): for sperm mobility 0.07 µg/L and for the number of spermatozoids 0.26 µg/L. The BMDL for sperm mobility was selected as the critical endpoint because this was the lowest BMDL. However, ANSES decided that the BMDL could not be used, based on criteria (BMD/BMDL ratio should be smaller than five) that can be found at the following website: https://bmds.readthedocs.io/en/latest/logic.html#bin-recommendation-logic.
It is not clear for FO what the status or origin of this website is.

Proposed BMD analysis of MCLR
We re-analysed the data, with the following adjustments:
1. The BMR was derived based on the BMR 1 SD approach, but performed on the log-scale. This is substantiated in Slob (2017). This approach has not yet been

implemented in the EFSA webtool but is implemented in the tools available at the RIVM website[2].

2. Since the groups of mice that have been exposed for 3 or 6 months are different animals (resulting in independent observations), study duration was included as a covariate in the BMD analysis.
3. The BMD confidence intervals (CIs) were based on model averaging (using 200 bootstrap runs), according to EFSA guidance;
4. We included four additional endpoints from Chen et al. (2011). The re-analysis was performed on the tabulated numerical data as provided in the paper. Therefore, the endpoints included in our BMD analysis are: number of spermatozoids, sperm mobility, sperm head abnormalities and plasma levels of testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH).

The BMD confidence intervals (CIs) are expressed as the range between the lower confidence limit (BMDL) and the upper confidence limit (BMDU). The BMD CIs for these five endpoints are shown in Figure 1, for the 3 and 6 month cohorts (group 3m and group 6m, respectively). The numbers behind the endpoints on the right side of the plots are the BMRs calculated from the BMR 1 SD (on log scale), expressed as a fractional change.

FO noticed that the relevance of these exposure durations (3 or 6 months) for exposure to humans and/or farm animals should be considered when deriving a health based guidance value for MCLR in the light of the anticipated duration of the exposure of humans (or animals). The anticipated duration of the exposure may be influential to the selection of the key toxicological endpoint.

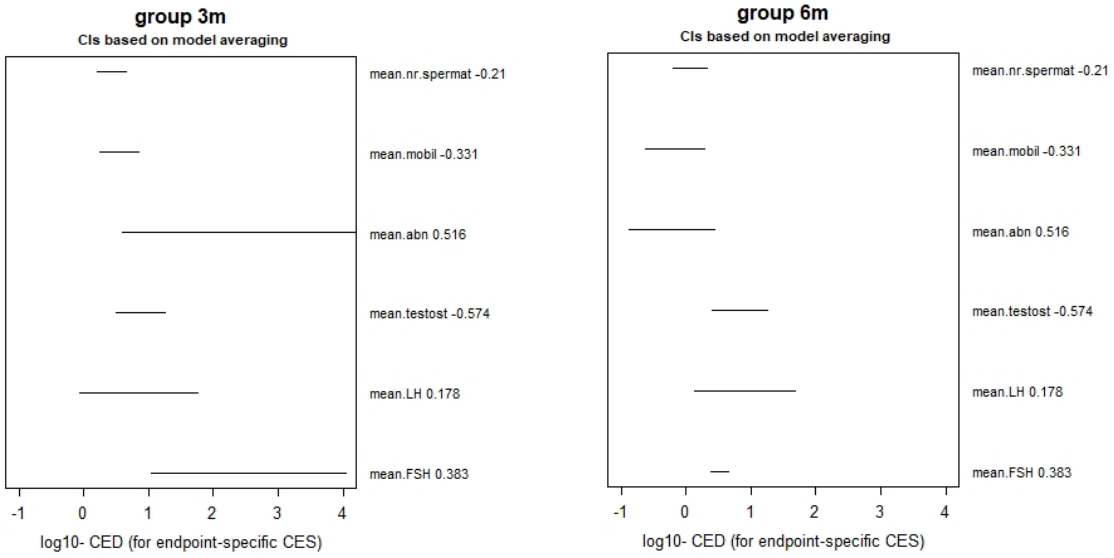

Figure 1. BMD confidence intervals for five endpoints reported by Chen et al. (2011), after three (left panel) and six (right panel) months of exposure. The numbers behind the endpoint names indicate the BMR (fractional change) calculated from the BMR 1 SD on log-scale.

Except for the endpoints sperm head abnormalities ("abn" in the graph) and FSH after 3 months of exposure, the CIs are in the same range, and roughly overlap each other. Most

[2] URL: https://www.rivm.nl/en/proast

of these BMD CIs span less than a factor of ten, which is a reasonable precision. The exact CIs are given in table 1.1 of Annex 1. The lowest BMDL is 0.13 µg/L (for endpoint sperm head abnormalities after six months of exposure). The associated BMDU was 2.7 µg/L. The dose-response data for this endpoint with one of the fitted models are shown in figure 2.1 of Annex 2.

Conclusion for MCLR

The data from Chen et al. (2011) result in BMD confidence intervals which are suitable for the selection of a BMDL. The lowest BMDL, from six endpoints considered, was 0.13 µg/L drinking water.

BMD analysis of CS

The key study that ANSES used for the derivation of the PoD of CS is by Chernoff et al. (2018). Chernoff et al. studied hepatotoxic and nephrotoxic effects in mice after subchronic oral exposure (90 days) to purified CS at exposure levels from 75 to 300 µg/kg bw/day. ANSES selected relative liver weight as the critical endpoint for BMD analysis. ANSES used a BMR of 5% (the default according to EFSA guidance) and found that the BMD (point estimate) was around 2 µg/kg bw, which is considerably lower than the lowest dose tested (75 µg/kg bw). As a 5% change in relative liver weight may be toxicologically considered as a very small effect, they also applied a BMR of 10%. This resulted in a BMD (point estimate) of around 10 µg/kg, which they still considered too far below the lowest dose tested. Based on the argument that low-dose extrapolation is not reliable, they rejected the results of the dose-response modelling for further risk assessment.

Proposed BMD analysis of CS

We re-analysed the data, with the following adjustments:

1. The BMR was derived based on the BMR 1 SD approach, but performed on the log-scale. For substantiation see Slob et al. (2017);
2. The study of Chernoff et al. (2018) consisted of two groups of mice, male (m) and female (f) and we used gender as a covariate in our BMD analysis;
3. The BMD confidence intervals (Cis) were based on model averaging (using 200 bootstrap runs), according to EFSA guidance;
4. In addition, we have also analysed nine other endpoints from Chernoff et al. (2018). This study is an OECD 408 guideline-compliant 90-day oral toxicity study that included all conventional parameters plus several additional endpoints (e.g. some for gene expression). The parameters used for the current dose response analysis were selected on the basis of visual inspection of the data, and in concordance with the analysis of the study authors that liver and kidney damage were the key toxic effects. Thus, the endpoints included in our BMD analysis are: alkaline phosphatase (ALP), alanine aminotransferase (ALT), blood urea nitrogen (BUN), cholesterol (Chol), triglycerides (TRG), body weight (BW), absolute liver weight, relative liver weight, absolute kidney weight and relative kidney weight.

The BMD CIs for these ten endpoints are shown in Figure 2, for male mice (group m) and female mice (group f). The numbers behind the endpoints on the right sight of the plots are the resulting BMRs (fractional change) calculated from the BMR 1 SD (on log-scale).

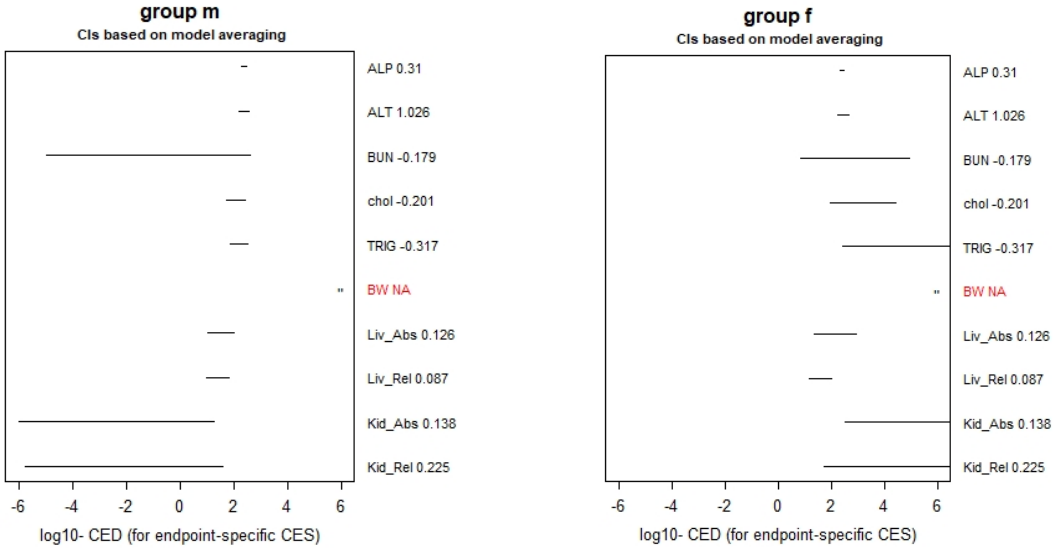

Figure 2. BMD confidence intervals for ten endpoints reported by Chernoff et al. (2018), for male (left panel) and female mice (right panel). The numbers behind the endpoint names indicate the BMR (fractional change) that was calculated from the BMR 1 SD on log-scale. Body weight (BW) did not show a significant trend with dose, therefore no BMD CI was calculated.

The BMD Cis for the male mice are in the same range, except for BUN and (absolute and relative) kidney weight. Some of the Cis for the female mice show a larger uncertainty, in a higher dose range: these endpoints did not show very clear effects. The exact Cis are given in table 1.2 of Annex 1. The kidney effects were ignored, as these data resulted in an extremely wide BMD confidence interval (7 to 8 orders of magnitude). The lowest BMDL is found for relative liver weight in the male group: 9.39 µg/kg bw. The associated BMDU was 64.4 µg/kg bw. The dose-response data for this endpoint with one of the fitted models (for each sex) is given in figure 2.2 of Annex 2.

Conclusion for CS

The data from Chernoff et al. (2018) result in acceptable BMD confidence intervals for 6 out of 10 endpoints and therefore these data are suitable for the selection of a critical PoD. The lowest BMDL is found for relative liver weight in the male group: 9.4 µg/kg bw.

Answers to questions

Question 1

Can, by application of Dose-Response modelling, Point of Departures be derived from the studies by Chen et al. (2011) and Chernoff et al. (2018)?

Answer 1

From the data on MCLR reported by Chen et al. (2011), as well as the data on CS reported by Chernoff et al. (2018) PoDs can be derived.

Question 2

Which PoDs can be derived for microcystin-LR (MCLR) and cylindrospermopsin (CS)?

Answer 2

For MCLR the BMDL was 0.13 µg/L (related to 'sperm head abnormalities'). For CS the BMDL was 9.4 µg/kg bw (related to 'relative liver weight'). Provided the studies by Chen and Chernoff are the key toxicity studies for these two cyanotoxins, the BMDL values derived from these studies are suitable PoDs for toxicological risk assessments.

Comments related to the ANSES assessment

MCLR analysis

1. The criterion that the BMD/BMDL ratio should be smaller than five (according to one of the criteria cited by ANSES from the aforementioned website[3]) is not in line with the EFSA guidance. EFSA considers the BMDU/BMDL ratio as the more appropriate ratio to be used as a measure for the uncertainty (imprecision) in the estimated BMD. However, EFSA does not provide strict criteria for acceptable precision, as this depends on the purpose of the assessment.
2. It is a general rule that a dose with a small effect size will be more uncertain than a dose with a larger effect size. Therefore, when the dose with an effect of 14.5% (the BMR used by ANSES for the number of spermatozoids) is too uncertain to be used for risk assessment, then the NOAEL cannot be an alternative: the uncertainty in the NOAEL will even be greater, as the effect size at the NOAEL is assumed to be zero. If the uncertainty in the BMD is considered too large to justify the use of the BMDL as a basis for risk assessment, then the logical consequence is to discard the specific dataset as a basis for risk assessment.
3. ANSES uses the BMR 1 SD approach based on the SD on the original scale. However, Slob (2017) showed that the BMR 1 SD is only valid when the SD of the log-transformed responses is used. When doing that for the two endpoints considered here, the BMR (as a percent change) was found to be larger: 21% and 33% for number of spermatozoids and sperm mobility, respectively. In general, the uncertainty in the associated BMDs tends to be smaller with larger BMRs.
4. ANSES did not use the data for 6 months exposure duration. However, these dose-response data seem to show similar effects at somewhat lower doses than the data for the three months exposure. There is no obvious reason not to include the data from the 6 month exposure in the assessment. The PROAST software enables such a combined dataset using exposure duration as a covariate in the BMD analysis.
5. ANSES did not use model averaging, the preferred approach according to EFSA. It should be noted, however, that this approach was not yet implemented in the software at the time ANSES performed their assessment.

CS analysis

1. The rather uncertain BMD10 that was originally found by ANSES for relative liver weight is due to the fact that the underlying data provide little information on the BMD. This is illustrated as example in figure 3, where the best-fitting curve indicates one possible location of the BMD10 (a default value of 10 was used for the critical effect size by ANSES). However, many other curves that describe the datapoints nearly equally well (not shown in figure 3) are possible in this case, leaving the location of the true BMD10 uncertain. This uncertainty is reflected by the width of the BMD confidence interval (1.9 – 33 µg/kg), as found by ANSES. However, in our view, this uncertainty is not unusual and no reason to reject the BMDL.
2. Using the BMDL of 1.9 µg/kg might be rather conservative, as the true BMD could also be close to the upper bound of the BMD confidence interval (33 µg/kg). Therefore, the discussion if this BMDL should form the basis of further risk assessment is legitimate. However, it is not justified to replace the BMDL by a LOAEL, divide that value by three, and consider the resulting dose as a dose without any effect. As the BMD analysis showed, the dose with a 10% effect was found to be somewhere between a factor of 2.3 to 40 lower than the lowest dose tested (= LOAEL = 75 µg/kg)[4]. Clearly, using a point of departure which is a

[3] URL: https://bmds.readthedocs.io/en/latest/logic.html#bin-recommendation-logic.
[4] Obtained by dividing the lowest dose by the BMDU and the BMDL, respectively.

factor of 3 below this LOAEL could well result in a severe underestimation of the toxic potency of the substance and thus in an underestimation of the actual risk.

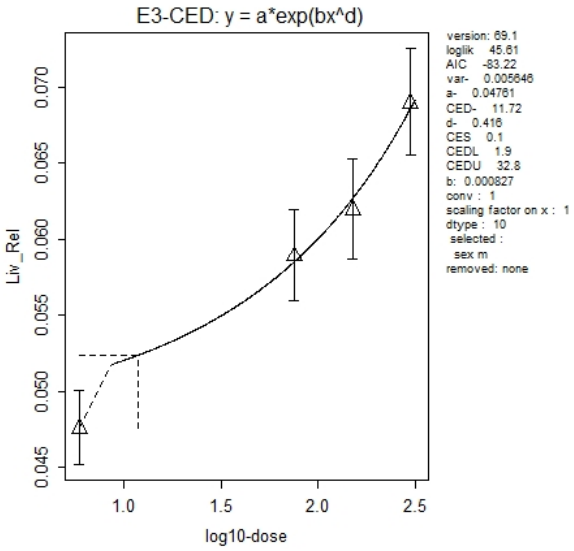

Figure 3. Dose-response data for relative liver weight from Chernoff et al. (2018), with the fitted exponential model. The horizontal dashed line indicates the level of a 10% increase (as used by ANSES), the vertical dashed line the associated BMD10. Note that the LOAEL divided by three is 25 µg/kg which is equal to 1.4 on the log-dose scale.

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ANNEX 1. Confidence intervals for MCLR and CS as derived by RIVM

Table 1.1. CIs for MCLR as derived by RIVM, based on Chen et al. (2011).

Endpoint	Exposure duration			
	3 months		6 months	
	BMDL	BMDU	BMDL	BMDU
nr of spermat	1.59	4.44	0.643	2.11
sperm mobil	1.74	7	0.239	1.88
sperm head ab-norm	3.93	Inf	0.129	2.71
testosterone	3.15	17.7	2.54	18.3
LH	0.87	56.6	1.35	48
FSH	11.2	11100	2.39	4.51

Table 1.2. CIs for CS as derived by RIVM, based on Chernoff et al. (2018).

Endpoint	Gender			
	Female mice		Male mice	
	BMDL (f)	BMDU (f)	BMDL (m)	BMDU (m)
ALP	214	310	191	314
ALT	179	468	159	380
BUN	7.43	84400	1.11E-05	407
chol	89.9	28100	57.3	259
TRIG	266	2.38E+14	78.6	330
BW	-	-	-	-
Liv_Abs	23.3	876	11	106
Liv_Rel	15	103	9.39	64.4
Kid_Abs	334	9.02E+30	1.02E-06	18.3
Kid_Rel	55.8	8.95E+58	1.74E-06	37.8

ANNEX 2. Dose-response data and fitted models for the endpoints with the lowest BMDL

Microcystine-LR

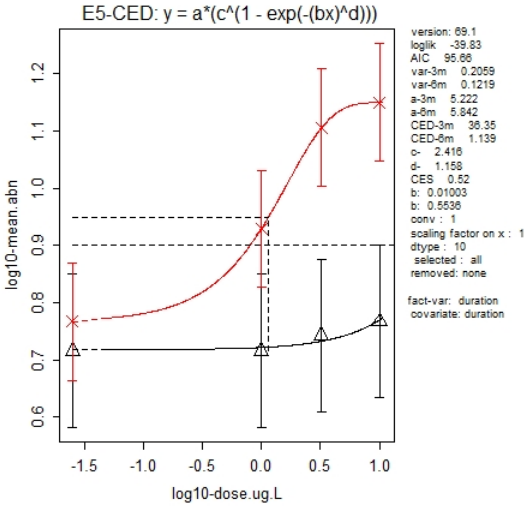

Figure 2.1. Mean sperm head abnormalities, with 95% confidence intervals, as a function of dose. Triangles three months; crosses: six months of exposure. Data from Chen et al. (2011). The two curves reflect the fitted exponential model, where exposure duration (3 or 6 months) was used as a covariate. The dashed horizontal lines indicate the BMR of 52%, the vertical dashed lines the BMDs for each exposure duration. Note that on the log-dose scale the controls should be imagined to be located at minus infinity, hence the dashed lower parts of the curves.

Cylindrospermopsin

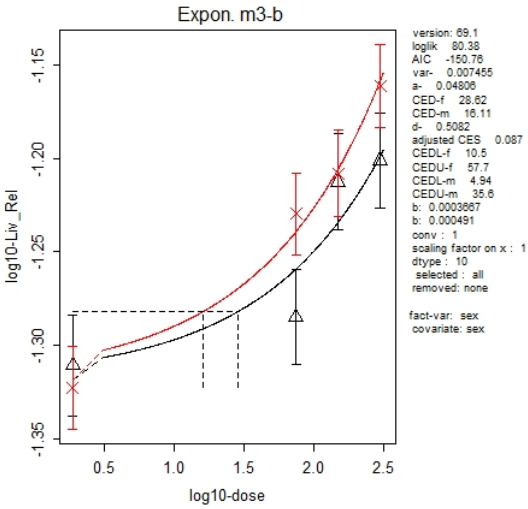

Figure 2.2. Mean relative liver weights with 95%-confidence intervals as a function of dose for males (crosses) and females (triangles). Data from Chernoff et al. (2018). The two curves reflect the fitted exponential model for each sex (used as a covariate in fitting the model).