National Institute for Public Health and the Environment
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FRONT OFFICE FOOD AND PRODUCT SAFETY

Derivation of an acute reference dose for the cyanotoxin cylindrospermopsin

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Afleiding van een acute referentiewaarde voor het blauwalgtoxine cylindrospermopsine

Samenvatting

Bureau Risicobeoordeling & onderzoek (BuRO) heeft het Front Office Voedsel- en Productveiligheid (FO) gevraagd om een acute referentiewaarde voor het blauwalgtoxine cylindrospermopsine (CS) af te leiden omdat dit toxine is aangetroffen in mosselen en oesters uit 2019. Het FO heeft een literatuuronderzoek uitgevoerd naar de (sub)acute toxiciteit van CS en heeft op basis van een aantal wetenschappelijke studies met proefdieren, die oraal waren blootgesteld aan CS, een acute referentiewaarde afgeleid.
De acute toxiciteit van CS werd onderzocht in muizen bij eenmalige doseringen van 1000 tot en met 8000 µg/kg lichaamsgewicht (lg). In de subacute studies werden muizen of ratten dagelijks gedurende enkele dagen of enkele weken oraal blootgesteld aan lagere doseringen (8-300 µg/kg lg/dag).
Bij eenmalige, hoge doseringen werden vanaf 1000 µg/kg lg schadelijke effecten waargenomen die bij hogere doseringen in ernst toenamen. Vanaf 2000 t/m 4000 µg/kg lg werd al sterfte waargenomen bij een deel van de dieren die waren blootgesteld. Bij een dosis van 8000 µg/kg lg stierven alle muizen binnen 1 tot 2 dagen. De schadelijke effecten werden voornamelijk waargenomen in lever en nieren van de proefdieren. De laagste dosering (van 1000 µg/kg lg) is door het FO als een zogenoemde acute Lowest Observed Adverse Effect Level (LOAEL) geduid. Bij meermalige, lagere doseringen werden al vanaf 8 µg/kg lg/dag effecten waargenomen die echter niet schadelijk waren. Vanaf 150 µg/kg lg/dag werd bij muizen vetinfiltratie in de lever aangetoond. Dit schadelijke effect was ook waargenomen in de studies naar de acute toxiciteit. Mede door de overeenkomst(en) in schadelijke effecten in de acute en subacute studies werd deze vetinfiltratie door het FO beoordeeld als een kritisch, schadelijk effect. Bij een orale dosering van 50 µg/kg lg/dag werden bij muizen na 2 weken blootstelling geen schadelijke effecten waargenomen. Deze dosering is als een zogenoemde subacute No Observed Adverse Effect Level (NOAEL) aangemerkt.
Op basis van bovengenoemde studies heeft het FO voor de mens een acute referentiewaarde afgeleid, een zogenoemde Acute Reference Dose (ARfD). Om de acute LOAEL en de subacute NOAEL waargenomen in muizen te kunnen extrapoleren naar een ARfD voor de mens zijn zogenoemde onzekerheidsfactoren (uncertainty factors, UFs) toegepast. Gelet op de onzekerheden in de acute studies in muizen is de NOAEL van 50

µg/kg lg uit de subacute studie genomen als vertrekpunt (Point of Departure, PoD) voor het afleiden van de ARfD. Gebruik makend van een standaard UF van 100, voor verschillen tussen mensen en dieren en voor verschillen tussen mensen onderling, is een ARfD afgeleid van 0,5 µg/kg lg.

Subject

The cyanotoxin cylindrospermopsin was found in several samples of mussels and oysters collected in 2019. The Office for Risk Assessment & Research (BuRO) would like to perform a risk assessment for these samples. Since no acute reference dose (ARfD) is available in the scientific literature, BuRO requested the Front Office Food and Product Safety (abbreviated as FO) to derive an ARfD for cylindrospermopsin.

Question

1. Is it possible to derive an acute reference dose (ARfD) for cylindrospermopsin?
2. If yes, what is the numerical value of this ARfD?

Conclusions

1) The toxicological data on the acute toxicity of CS are quite limited. Preferably, an ARfD is derived from single dose toxicity studies. However, in view of the uncertainties in the single dose studies available, the ARfD was based on subacute toxicity data based on similar effects as observed after acute exposure.

2) An ARfD of 0.5 µg/kg bw is established, based on a NOAEL of 50 µg/kg bw/day obtained from a subacute toxicity study in mice. The critical effect was lipid infiltration in liver cells. To this NOAEL an uncertainty factor of 100 was applied.

Introduction

During warm periods, blooms of cyanobacteria (blue-green algae) can be observed in surface waters. Cyanobacteria can produce toxic substances (cyanotoxins) which can end up in the water. Consumption of contaminated food (e.g. shellfish and crustaceans) or drinking water could be harmful to humans.
This toxin has been detected by Wageningen Food Safety Research (WFSR) in Dutch mussels and oysters collected in 2019 and analysed in 2020. This evaluation addresses the (sub)acute toxicity of cylindrospermopsin (CS) to find out whether it is possible to derive an ARfD.

CS inhibits protein synthesis and is toxic to a variety of tissues/organs: liver, kidneys, spleen and lungs, it may affect spermiogenesis and is suspected of genotoxicity.
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) published a risk assessment report on CS (ANSES 2019a,b). ANSES derived for this toxin a subchronic toxicological reference value (TRV) for the oral route[1]. The TRV for CS amounted to 0.14 µg/kg bw/day. The TRV for CS was derived from a Lowest Observed Adverse Effect Level (LOAEL) for adverse liver effects observed in a 90-day oral toxicity study in mice, published by Chernoff et al. (2018).
In May 2020, by request of BuRO, RIVM has performed Bench Mark Dose (BMD) analyses for CS (RIVM, 2020). Starting point for the assessment of CS has been the assumption that the study by Chernoff et al. (2018) is the critical study for the derivation of a Point of Departure (PoD) for a (sub)chronic Health Based Guidance Value (HBGV) for CS. Based on this subchronic study, a Bench Mark Dose Lower Confidence Limit (BMDL) of 9.4

[1] In the previous evaluation (RIVM, 2020), this toxicological reference value was erroneously referred to as 'tolerable daily intake'.

µg/kg bw (related to an 8.7%[2] increase in 'relative liver weight') was derived (RIVM, 2020).
In addition, in November 2019, the World Health Organization (WHO) published a draft background document on CS for the development of Guidelines for drinking water quality and Guidelines for safe recreational water environments (WHO, 2019a). At the moment, the WHO report is still a draft version open for public review.
ANSES (2019) and WHO (2019a) did not address the possibility and need to derive an ARfD for CS.

Methodology / approach
First, a literature search was performed to identify scientific information relevant for the derivation of an oral ARfD for CS. Preferably, an ARfD is derived from a single dose study in animals and/or humans based on (the absence of) acute adverse effects. Alternatively, short-term (multiple dose) studies may be used describing subacute adverse effects in animals and/or humans. In principle, subchronic toxicity studies are not used to derive an ARfD but these studies were also taken into account to compare adverse effects observed in these studies with effects observed in (sub)acute toxicity studies. Due to the possible source of contamination (food and/or water) only oral exposure to CS was considered relevant. Next, on the basis of this toxicological information (see section on toxicology) it was investigated whether the collected information was suitable to derive an ARfD (see section on the derivation of an ARfD).

Literature search
Due to the fact that there was little information on the (sub)acute toxicity and (suspected) genotoxicity of CS in the reports of ANSES and WHO, a literature search was carried out to collect new information that recently may have been published. To this end, a literature search was conducted in Embase, Pubmed and Scopus using "cylindrospermopsin" as search term (in title, abstract, keywords). This gave 523 hits in Pubmed, 819 hits in Scopus and 625 hits in Embase. The references retrieved from searching the three databases were extracted and combined into an Endnote file and duplicates were removed. In total 845 unique references were found. Based on title screening, a first selection of possibly relevant articles was made. Initially, animal studies, *in vitro* genotoxicity data, human data and reviews on the toxicity of CS were included. This led to the inclusion of 171 references for the next selection step. References that were not already included in the reports from WHO (2019a) or ANSES (2019), were screened based on abstract and if necessary the full text, to assess their relevance with respect to the (sub)acute toxicity of CS. Recent reviews were used to crosscheck that no key references were missing. The results showed that recently several new genotoxicity studies, conducted according to guidelines of the Organization for Economic Cooperation and Development (OECD), were published. Because genotoxicity is mainly of relevance for assessing the risks of (sub)chronic exposure to CS, it was decided, in consultation with BuRO, not to assess the genotoxicity of CS in the present evaluation. In addition to those already cited by ANSES (2019) and WHO (2019a) two studies on subchronic toxicity were included in the present assessment.

Analysis of toxicological data
According to the WHO, when assessing the need for an ARfD the entire toxicity database should be reviewed, including all available information on the effects of human exposure

[2] The BMR was derived based on the BMR 1 SD approach, but performed on the log-scale. For substantiation see Slob et al. (2017)

(WHO, 2019b). In concordance with WHO we have focused on the results from acute, subacute, subchronic and developmental toxicity studies and any human data.

Acute toxicity

Three studies have investigated the acute oral toxicity of extracts of *Cylindrospermopsis raciborskii* containing CS, but these studies have not quantified the concentration of CS in the extract, with consequential limitations for the estimation of the actual exposure and the estimation of the endpoint, i.e. LD_{50} (Falconer et al., 1999; Shaw et al., 2000; Falconer & Humpage, 2001). Three other studies have used either freeze-dried algal culture with a quantified concentration of CS (Seawright et al., 1999), cell-free extracts and CS purified from these extracts (Shaw et al., 2001) or 98% pure CS (Bazin et al., 2012). In a study with male MF1 mice, 18 animals were dosed in pairs by gavage at dose rates ranging from 2500 to 8300 µg/kg bw of CS equivalents[3] (eq). At the lowest dose, one mouse appeared grossly normal and the other mouse showed foamy hepatocyte cytoplasm due to fatty infiltration. The lowest lethal dose of extracts of *C. raciborskii* strains was 4400 µg/kg bw as CS equivalents, the highest non-lethal dose was 6900 µg/kg bw, suggesting that the median lethal dose was in the range of 4400–6900 µg/kg CS equivalents. Death occurred 2 to 6 days after dosing and pathological changes included marked fatty liver, acute renal tubular necrosis, atrophy of the thymic cortex and the lymphoid follicles in the spleen, subepicardial and myocardial hemorrhages, and multiple ulcerations of the esophageal part of the gastric mucosa (Seawright et al., 1999). In a study of Shaw et al. (2001) Quackenbush mice (four per dose) were given single oral doses of *C. raciborskii* extract at 1000, 2000, 4000, 6000 and 8000 µg CS equivalents/kg bw. Mortality occurred in 2/4 mice at 6000 µg CS equivalents/kg bw (in 5 days) and 4/4 mice at 8000 µg CS equivalents/kg bw (in 24–48 hours). Lipid infiltration and cell necrosis was observed in the liver of the animals at these doses. At 1000, 2000 and 4000 µg /kg bw an increasing degree of foamy hepatocellular cytoplasm, due to fatty infiltration, was observed (Shaw et al., 2001). Bazin et al. (2012) exposed male Swiss albino mice (three per dose) by gavage to CS (98% purity) at dose levels of 1000, 2000 and 4000 µg/kg bw as part of a comet assay and a micronucleus assay. The mice were killed after 24 hours. One out of three mice died before scheduled necropsy at a dose of 2000 µg/kg bw. One out of three mice administered 4000 µg/kg bw was moribund after 24 hours and severe adverse effects were observed in liver and intestines in another mouse administered 4000 µg/kg bw as well. Histological analyses revealed apoptosis in the liver and kidney of mice exposed to 2000 and 4000 µg/kg bw. At these same doses, apoptosis of lymphocytes in Peyer's patches of the duodenum and jejunum was demonstrated. At 1000 µg/kg bw no effects were seen in the liver and some isolated apoptotic cells in the kidney were observed.

From the three studies with quantified dose levels of CS an LD_{50} of approximately 4000 to 6000 µg/kg bw can be deduced. At a dose of 1000 µg/kg bw some foamy hepatocellular cytoplasm was observed with cell-free extracts (Shaw et al., 2001) and no effects in the liver and some apoptosis in the kidney were observed after dosing with 98% pure CS (Bazin et al., 2012). A short summary of the above-mentioned relevant studies with acute single dose oral exposure to CS (pure or in cell-free extracts) is given in table 1.

[3] the amount of dried algal culture administered was equivalent to 2500 to 8300 µg CS/kg bw.

Table 1. Single oral dose studies with quantified CS (pure or in cell-free extracts) and the lowest observed effect levels and lowest lethal levels.

Species (gender)	Dose (µg CS eq/kg bw)	Purity	Observed effect	Mortality (x out of y)	Reference
Mice (male)	2500	Extract	foamy hepatocellular cytoplasm		Seawright et al. (1999)
	4400	Extract	necrosis of liver cells	1/2	
Mice (?)	1000	Extract	foamy hepatocellular cytoplasm		Shaw et al. (2001)
	6000	Extract	necrosis of liver cells	2/4	
Mice (male)	1000	98% pure	isolated apoptotic kidney cells		Bazin et al. (2012)
	2000	98% pure	apoptotic kidney and liver cells	1*/3	

* One animal died before the scheduled necropsy.

Subacute toxicity

A short-term study (14 days) was carried out by Shaw et al. (2001). Groups of Quackenbush mice (four per dose) were given purified CS from freeze-dried extracts of *C. raciborskii* at doses of 0 (control), 50, 150 and 300 µg/kg bw/day by gavage. The doses allowed the authors to identify a NOAEL of 50 µg/kg bw/day and a LOAEL of 150 µg/kg bw/day describing fatty infiltration in the liver as a critical effect. Lymphophagocytosis was observed at 300 µg/kg bw/day in the spleen.

In 2004, Reisner et al. conducted a 3-week study exposing 4-week-old male ICR mice daily to CS purified from *Aphanizomenon ovalisporum* via drinking water. A group of 8 mice was exposed to water ad libitum containing 600 µg/L of CS, equal to 66 µg/kg bw/day (based on the measured water consumption of 2.8 mL of water per day) and a control group of 8 mice received only untreated drinking water ad libitum. At autopsy, the weights of the liver and testes were higher in the treated mice than in the control group. An increase in hematocrit was observed after 21 days of exposure to CS as well as morphological changes in red blood cells. Exposure to 66 µg/kg bw/day also resulted in an increase in the level of cholesterol in the membranes of red blood cells and in the plasma as well as a decrease in cholesterol in the liver compared to the non-exposed group. Therefore, the LOAEL in this study was 66 µg/kg bw/day. However, the effects in this study were not reported in the single dose studies, contrary to the fatty infiltration. Therefore they seem not attributable to acute exposure per se and are not suitable for derivation of an ARfD (see also Solecki et al., 2005).

In a study of Díez-Quijada et al. (2019), male Wistar rats (5 per dose) were exposed to water (negative control) or 8, 23.7 or 75 µg/kg bw CS by gavage at 0, 24 and 45 hours as part of a genotoxicity study (combined micronucleus and comet assay). Clinical signs and bodyweight were recorded during the exposure period. Animals were killed 3 hours after the last administration (at t=48 h) and blood, liver and stomach samples were collected for histopathology. No mortalities or signs of toxicity were observed. No statistically significant changes in body weight or relative organ weight were recorded compared to the negative control group. However, significantly altered gastric mucus

secretion was noticed. Mucosal damage in the stomach was observed in all treated rats. In addition, at the two highest doses, the gastric mucosa exhibited alterations and the mucous content was decreased. According to the authors, the lesions in the stomach could be related to a possibly irritant effect of the cyanotoxin. The liver of treated rats (all groups) showed modifications in the nuclei structure, and an increase in endomitosis with the production of binucleated hepatocytes was seen. In addition, the liver of treated rats (all groups) showed proliferation of smooth endoplasmic reticulum with dilation of endoplasmic reticulum vesicles. At the low and mid dose, changes were found at perilobular hepatocytes while at the high dose, changes in both perilobular and centrilobular hepatocytes were observed. However, the observed changes in the liver were poorly described and no qualitative scores were given. In addition, histological changes such as apoptosis or necrosis were not reported. In view of this the FO assessed the observed effects as not suitable for the derivation of an ARfD.
A short summary of the above-mentioned relevant studies with subacute exposure to CS (a few days to a few weeks) is given in table 2. Unfortunately, the qualitative rather than quantitative description of the results from these studies, including the 14-day oral toxicity study in mice by Shaw et al. (2001) (mainly limited histopathological findings) prevented the analysis of the study data using dose-response modelling.

Table 2. Animal studies with subacute exposure to CS and observed NOAEL/LOAEL (in µg/kg bw).

Species	Exposure duration	Critical effect	NOAEL/LOAEL (µg/kg bw)	Reference
Rats	2 days	Polyploid hepatocytes	Not applicable	Díez-Quijada et al. (2019)
Mice	2 weeks	Lipid infiltration in liver	NOAEL = 50	Shaw et al. (2001)
Mice	3 weeks	Liver weight and cholesterol	LOAEL = 66	Reisner et al. (2004)

Subchronic toxicity
In this section the subchronic toxicity is briefly mentioned with the sole purpose to check whether critical effects observed after subchronic exposure to animals are in line with adverse effects observed after (sub)acute exposure.
Humpage and Falconer (2003) performed a study in which male Swiss Albino mice were exposed for 11 weeks to CS purified from an extract of *C. raciborskii* cells (purity not specified). The mice received a daily dose by gavage of 0, 30, 60, 120 and 240 µg/kg bw/day. Compared to the control group, a significant increase in the relative weight of the kidneys and the absolute weight of the testes was noted at a dose of 60 µg/kg bw/day as well as an increase in the relative weight of the liver at 240 µg/kg bw/day of exposure. Regarding clinical chemistry parameters, no statistically significant changes were observed in serum bilirubin and serum bile acids. Other effects observed included decreased urinary protein and creatinine concentrations (≥ 120 µg/kg bw/day), minor liver histopathology (not specified) at doses ≥ 120 µg/kg bw/day and decreased urine specific gravity and renal proximal tubular damage (at 240 µg/kg bw/day). Based on the results, the authors established a NOAEL of 30 µg/kg bw/day, considering the increase in kidney weight as a critical effect.
In a study of Sukenik et al. (2006), male and female mice were given CS-containing *A. ovalisporum*-free culture medium as their drinking water for up to 42 weeks (Sukenik et al., 2006). By the 20th week of exposure, half of the mice in each group were euthanised, while the other half were exposed 22 more weeks before euthanasia. In

addition, the CS concentration in the medium gradually increased from 100 to 550 µg/L over the dosing period so that the estimated daily CS doses increased from 10 µg/kg bw to 55 µg/kg bw over the 42 weeks. Therefore, although this study is giving supporting, qualitative evidence for CS toxicity in kidney and liver, the experimental design (in particular the dosing and the dosing period) makes this study unsuitable for the derivation of an ARfD.
Chernoff et al. (2018) performed an oral 90-day study in which mice were exposed to purified (>95%) CS at 0, 75, 150 or 300 µg/kg bw/day via gavage. The study was conducted according to OECD guideline 408 (OECD, 2018). Several additional endpoints (e.g. some for gene expression) were included. RIVM (2020) performed a BMD analysis on the results of this study. The parameters used for the 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. The BMDL of 9.4 µg/kg bw, related to an 8.7% increase in relative liver weight in male mice, was selected as possible PoD for a (sub)chronic health based guidance value for CS from this study (RIVM, 2020).

Reproductive and developmental toxicity
Repeated oral exposure to low doses of CS (up to 3 µg/kg bw per day) during gestation days (GD) 1 – 20 did not show any developmental effects in rats (Sibaldo de Almeida et al., 2013). High maternal toxicity was observed in developmental toxicity studies in which CS was administered intraperitoneally at a dose of 50 µg/kg bw per day (Rogers et al., 2007 as cited by ANSES, 2019; Chernoff et al., 2011, as cited by ANSES, 2019; Chernoff et al., 2014, as cited by WHO, 2019a), but it is considered likely that this is due to the route of administration which is not relevant for oral exposure. ANSES (2019) concluded that not sufficient *in vivo* oral data were available to characterize the effects of CS on reproduction and development. According to WHO (2019a), no effects were seen on fetus weight, mortality, skeleton or soft tissues in Rogers et al. (2007) and the surviving pups from animals dosed during GD 13-17 had reduced birth weight and postnatal growth compared to the pups from the controls animals or pups from animals dosed during GD 8-12.

In the literature search, no additional *in vivo* studies on reproductive and developmental toxicity were found, except for two studies in zebra fish. These were not considered to be of relevance for the derivation of an ARfD and therefore were not included in this evaluation.

Human data
No human data on the toxicity of CS were included by ANSES (2019). WHO (2019a) describes the involvement of cyanobacterial toxins, including CS, in algal bloom-associated human disease outbreaks from municipal water supplies. CS was detected in water in 2 of 11 algal bloom-associated disease outbreaks among users of freshwater lakes in 2009 and 2010 as described by the US Centers for Disease Control and Prevention. However, the co-occurrence of other cyanotoxins did not allow to estimate the relative contribution of CS to the multiple symptoms (nausea, vomiting, diarrhoea, abdominal cramps, anorexia) (Hilborn et al., 2014 as cited by WHO, 2019a). The level of exposure to CS associated with these outbreaks is not known. No further human data on CS were found in our literature search.

Derivation of an acute reference dose
The use of particular toxicological end-points that are most relevant to establishing ARfDs have been reviewed by Solecki et al. (2005) and WHO (2019b) and its suitability for CS

toxicity will be described underneath. The use of the applied uncertainty factors (UFs) to account for extrapolation uncertainties and study limitations, will be explained in the subsequent section.

Biological and toxicological considerations

As stated before, an ARfD is derived from a single dose study in animals and/or humans based on (the absence of) acute adverse effects. Alternatively, short-term (multiple dose) studies describing subacute adverse effects in animals and/or humans may be used as supporting evidence. Solecki et al. (2005) and WHO (2019b) have described the relevance of adverse effects observed after repeated-dose exposure to pinpoint particular toxicological end-points that are relevant to establish an ARfD.

Based on studies with extracts containing CS, the estimated LD_{50} of CS is about 4000–6000 µg /kg bw in mice. A lethal dose of CS showed effects on the liver, spleen, thymus, heart, esophagus and gastric mucosa (Seawright et al., 1999). Shaw et al. (2001) observed fatty infiltration and cell necrosis in the liver at doses of 4000 µg /kg bw and higher. Bazin et al. (2012) demonstrated, 24 h after CS administration to mice, necrosis of kidney and liver cells and mortality. In a short-term study (14 days) in mice, carried out by Shaw et al. (2001), lymphophagocytosis in the spleen and lipid infiltration in the liver was observed.

Taking acute effects into consideration we consider the foamy hepatocellular cytoplasm, due to fatty infiltration, and necrosis in kidney and liver cells relevant effects for the derivation of an ARfD. After oral administration of a single, sublethal dose of 1000 µg CS/kg bw these effects have been observed (to a small degree) in mice with a cell-free extract (Shaw et al., 2001) and 98% pure CS (Bazin et al., 2012), see also table 1. These effects were substantiated in a short-term study (14 days) in mice, where fatty infiltration has been observed at an oral dose of 150 µg/kg bw/day but no adverse effects were observed at 50 µg/kg bw/day (Shaw et al., 2001), see also table 2.

Summarised, from the information provided by ANSES (2019) and reported above, it is clear that the liver is a target organ for acute CS toxicity. Therefore, we conclude that the adverse effect of lipid infiltration in the liver of mice after a single dose or subacute exposure can be used to determine the PoD for the derivation of an ARfD for CS.

Uncertainty factors

Uncertainty factors (UFs) are used to extrapolate from animal data to the average human and to allow for variation in sensitivity within the human population. Usually, default factors of 10 are applied to account for inter- and intraspecies variabilities, resulting in a total assessment factor of 100. Additional factors may be applied to account for deficiencies in the toxicological database, e.g. to extrapolate from a LOAEL to a NOAEL etc.

Referring to the information summarised in table 1, a single oral dose of 1000 µg /kg bw in mice did not lead to mortality but (minor) adverse effects were observed in kidneys (Bazin et al., 2012) and liver (Shaw et al., 2001). Therefore this sub-lethal dose is considered the acute LOAEL. Besides the default overall factor of 100 (for intra- and interspecies variability), the application of an additional UF related to this single, sub-lethal dose in mice is justified (ECHA, 2012). In case of CS, this additional UF accounts for the following items: 1) the steepness of the dose-effect relationship; 2) the severity of the effect; 3) the limited number of animals (3 or 4 per dose); 4) the use of a single sex of a single species (male mice); 5) the qualitative histopathological interpretation and 6) the absence of an acute NOAEL. However, the magnitude of this additional UF has not been specified by ECHA and should be determined on a case by case basis (ECHA, 2012). In our view, items #1-5 justify an additional UF of 10. In addition, the dose of 1000 µg /kg bw should be treated as an acute LOAEL. The lack of an acute NOAEL would

justify an additional UF and it has been suggested by ECHA to use an assessment factor between 3 (as minimum in the majority of cases) and 10 (as maximum in exceptional cases).

WHO also indicated that the toxic mode of action and toxicokinetics should be taken into consideration. ANSES has reported on these aspects and concluded that the information on mode of action and on toxicokinetics is very limited (ANSES, 2019). Hence, there are no good reasons to deviate from the default uncertainty factors used for extrapolating from animals to humans.

Different population subgroups

It is preferable to set a single ARfD to cover the whole population, in particular for risk management and enforcement purposes. However, it is important to ensure that an ARfD is adequate to protect the embryo/fetus from possible in utero effects. Information on reproductive and developmental toxicity of CS is limited, but in developmental toxicity studies with oral and intraperitoneal administration of CS no effects were observed that are likely to be elicited by a single dose of CS. Thus, an additional ARfD for women of childbearing age is not required.

Derivation of the ARfD

Based on the above, we conclude that the dose of 1000 µg/kg bw should be treated as an acute LOAEL and that the dose-effect curve is steep. Therefore, we consider it appropriate to apply an additional UF of 3 for the extrapolation from an acute LOAEL (1000 µg/kg bw) to NOAEL in combination a UF of 10 for study deficiencies and dose response characteristics. Application of the overall additional UF of 30 to the acute LOAEL of 1000 µg/kg bw provides a PoD of 33 µg/kg bw for the derivation of an ARfD. However, in the subacute toxicity study (14 days) carried out by Shaw et al. (2001) a NOAEL of 50 µg/kg bw/day has been observed which indicates that the additional UF of 30, applied to the LOAEL, may be (somewhat) conservative. In view of all uncertainties connected to the studies by Shaw et al. and Bazin et al., the NOAEL of 50 µg/kg bw/day from the subacute (14-day) study of Shaw et al. is preferred as a PoD. This PoD is slightly higher than the NOAEL of 30 µg/kg bw observed in a subchronic study in mice (Humpage and Falconer, 2003) and approximately 5 times higher than the BMDL of 9.4 µg/kg bw derived by the FO from a subchronic study in mice performed by Chernoff et al. (2018). Applying an overall uncertainty factor of 100, to account for intra- and interspecies differences, to this PoD leads to an ARfD of 0.5 µg/kg bw.

Conclusions

For the overall conclusions we refer to the questions asked by BuRO.

1. Is it possible to derive an acute reference dose (ARfD) for cylindrospermopsin?

The toxicological data on the acute toxicity of CS are quite limited. Preferably, an ARfD is derived from single dose toxicity studies. However, in view of the uncertainties in the single dose studies available, the ARfD was based on subacute toxicity data based on similar effects as observed after acute exposure.

2. If yes, what is the numerical value of this ARfD?

An ARfD of 0.5 µg/kg bw is established, based on a NOAEL of 50 µg/kg bw/day obtained from a subacute toxicity study in mice. The critical effect was lipid infiltration in liver cells. To this NOAEL an uncertainty factor of 100 was applied.

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