ANSES Opinion
Request No. 2016-SA-0298
Related requests: No. 2016-SA-0165 and No. 2015-SA-0207

The Director General

Maisons-Alfort, 7 January 2019

OPINION
of the French Agency for Food, Environmental and Occupational Health & Safety

on the proposed subchronic TRV by the oral route for cylindrospermopsin (CAS No. 143545-90-8)

ANSES undertakes independent and pluralistic scientific expert assessments.
ANSES primarily ensures environmental, occupational and food safety as well as assessing the potential health risks they may entail.
It also contributes to the protection of the health and welfare of animals, the protection of plant health and the evaluation of the nutritional characteristics of food.

It provides the competent authorities with all necessary information concerning these risks as well as the requisite expertise and scientific and technical support for drafting legislative and statutory provisions and implementing risk management strategies (Article L.1313-1 of the French Public Health Code).

Its opinions are published on its website. This opinion is a translation of the original French version. In the event of any discrepancy or ambiguity the French language text dated 7 January 2019 shall prevail.

On 19 July 2016, ANSES received a formal request from the Directorate General for Health (DGS) to update its work for the assessment of the risks related to the presence of cyanobacteria and their toxins in water intended for human consumption, and water for bathing and other recreational activities.

1. BACKGROUND AND PURPOSE OF THE REQUEST

In the context of the request concerning an assessment of the risks related to the presence of cyanobacteria and their toxins in water intended for human consumption, and water for bathing and other recreational activities, it became necessary to update the body of reference work concerning the toxicity of cyanotoxins.

Two toxins were identified by the experts as requiring specific work. In view of its ongoing mission to develop toxicity reference values (TRVs), and in order to meet the terms of this request, ANSES decided to develop a TRV for these two toxins: cylindrospermopsin (CAS No. 101043-90-8) [referred to as CYN in the following opinion] and microcystin-LR (CAS No. 143545-37-2). In line with its long-term mission, the Agency is publishing the result of this work in two separate and specific opinions.

The purpose of this Opinion is therefore to propose a subchronic TRV by the oral route for cylindrospermopsin (CYN), a toxin for which few studies have been conducted to date. This TRV will be adopted and used during the Agency's more general expert appraisal of the health risks related to the presence of cyanobacteria (Request No. 2016-SA-0165).

A toxicity reference value, or TRV, is a toxicological indicator for qualifying or quantifying a risk to human health. It establishes the link between exposure to a toxic substance and occurrence of an adverse health effect. TRVs are specific to a duration (acute, subchronic or chronic) and route (oral or respiratory) of exposure. The way TRVs are established differs depending on the knowledge or assumptions made about the substances' mechanisms of action. Currently, the default assumption is to consider that the relationship between exposure (dose), and effect (response) is monotonic. In the current state of knowledge and by default, it is generally considered that for non-carcinogenic effects, toxicity is only expressed above a threshold dose (ANSES, 2017).

In practice, establishing a TRV involves the following steps:

- identifying and analysing the available toxicity data, based on epidemiological and/or experimental studies;
- identifying the target organ(s) and critical effect;
- identifying the assumption according to which it is established: with or without a threshold dose, depending on the substance's mode of action;
- choosing a good quality scientific study generally enabling establishment of a dose-response relationship;
- defining a critical dose for humans or animals from this study and, if required, in the case of a critical dose obtained in animals, adjusting this dose to humans;
- for a threshold TRV, applying uncertainty factors to this critical dose so as to derive a TRV that is applicable to the entire population in question;
- for a non-threshold TRV, conducting a linear extrapolation to the origin in order to determine an excess risk per unit.

TRVs are established according to a highly structured and rigorous approach involving collective assessments by groups of specialists.

A literature search carried out via the Scopus and PubMed databases without date limits identified 2000 articles relating to the toxicity of CYN. Of these studies, three were considered relevant by the experts for the establishment of a subchronic oral TRV, and only one led to the establishment of a toxicity reference value (TRV) representative of exposure scenarios related to the ingestion of water intended for human consumption or water during recreational water activities. This Opinion therefore proposes a subchronic oral TRV related to the ingestion of CYN.

2. ORGANISATION OF THE EXPERT APPRAISAL

The expert appraisal was carried out in accordance with French Standard NF X 50-110 "Quality in Expert Appraisals – General Requirements of Competence for Expert Appraisals (May 2003)".

The collective expert assessment was carried out by the Expert Committee (CES) on "Health Reference Values". The methodological and scientific aspects of the work were presented to the CES. Two rapporteurs were appointed, one to evaluate toxicity studies on the genotoxic status of CYN and the second to evaluate studies eligible for the establishment of an oral subchronic TRV. The work was adopted by the CES on "Health Reference Values" at its meeting on 18 October 2018.

ANSES analyses the interests declared by the experts prior to their appointment and throughout the work, in order to avoid potential conflicts of interest with regard to the matters dealt with as part of the expert appraisal.
The experts' declarations of interests are made public via the website of the Ministry of Solidarity and Health (https://dpi.sante.gouv.fr).

3. ANALYSIS AND CONCLUSIONS OF THE CES

■ Summary of the toxicological data

The only toxicity data on CYN available to date are from animal studies.

- Toxicokinetics

No *in vivo* oral data were available to characterise CYN absorption after exposure.

Studies in which the toxin was administered orally and which show a systemic effect suggest absorption from the gastrointestinal tract (Humpage and Falconer, 2002-2003; Sukenik *et al.*, 2006; Chernoff *et al.*, 2018). *In vitro* studies have shown the passage of the molecule through human intestinal Caco-2 cells but the mechanism of entry into these cells has not been described (Fernandez *et al.*, 2014; Pichardo *et al.*, 2017).

After intraperitoneal exposure in rodents, CYN was detected in the liver, kidneys, heart, lungs, spleen, blood and bile. The highest levels were mainly detected in the liver and kidneys (Norris *et al.*, 2001).

CYN appears to be metabolised by the cytochrome P450 system (Runnegar *et al.*, 1995; Shaw *et al.,* 2000; Norris *et al.*, 2002; Froscio *et al.*, 2003). The presence of metabolites in the urine of exposed rodents has been shown but has not been characterised (Norris *et al.*, 2001).

CYN and its metabolites appear to be excreted mainly in urine and faeces, based on available intraperitoneal data (Norris *et al.*, 2001).

- Acute and subacute toxicity

During acute oral exposure of rodents to CYN, the main observations are:

- liver effects: hepatic cytolysis, infiltration of inflammatory cells and lipids (Shaw *et al.*, 2000, 2001; Bazin *et al.*, 2012);
- kidney effects: tubular necrosis, alteration of the proximal tubule, alteration of the glomeruli (Bazin *et al.*, 2012);
- more rarely, intestinal bleeding (Seawright *et al.*, 1999; Bazin *et al.*, 2012).

- Irritation and sensitisation

A single study showed irritant and sensitising effects following dermal exposure to CYN in mice (Stewart *et al.*, 2006). However, on the basis of a single study, it is not possible to conclude as to the effects of CYN regarding irritation and sensitisation.

- Subchronic and chronic toxicity

In rodents, CYN mainly induces:

- kidney effects: histological alteration, alteration of proximal tubules, nephromegaly (Humpage and Falconer, 2002; Sukenik *et al.*, 2006; Chernoff *et al.*, 2018);
- liver effects: hepatic cytolysis, inflammation, morphological and histological changes, hepatomegaly, pigmentation abnormalities (Humpage and Falconer, 2002; Sukenik *et al.*, 2006; Chernoff *et al.*, 2018);
- biochemical effects: increased serum levels of liver enzymes, decreased cholesterol and triglyceride levels (Humpage and Falconer, 2002; Chernoff *et al.*, 2018).

- Reprotoxicity and effects on development

No *in vivo* oral data were available to characterise the effects of CYN on reproduction and development.

Repeated intraperitoneal exposure to low doses of CYN (less than 3 $\mu g.kg^{-1}.d^{-1}$) did not show any developmental effects (Almeida *et al.*, 2013). The high maternal toxicity found in pregnant mice

exposed intraperitoneally to CYN can be explained by the mode of administration (Rogers *et al.*, 2007; Chernoff *et al.*, 2011).

- Genotoxicity

Based on the results of genotoxicity tests (micronucleus test, comet assay, Ames test, chromosomal aberration test, *etc.*), the results of *in vitro* studies support the existence of a genotoxic effect of CYN; the *in vivo* results remain very fragmented and only one gavage study showed positive results in the colon out of six organs investigated. Concerning the mode of action, while an aneugenic effect has been described, DNA damage has also been observed without its origin being clearly established.

- Carcinogenicity

To date, there is only one preliminary study investigating the carcinogenicity of CYN in orally exposed rodents (Falconer and Humpage, 2001). This study suggests an initiating effect of CYN: induction of neoplastic foci is observed mainly in the liver. An *in vitro* study (Maire *et al.*, 2010) confirms these observations by revealing changes in cell morphology due to the carcinogenic potential of CYN at very low doses. However, it is not possible to reach a decision on the potential carcinogenic effect of CYN, given the very limited data available. Further studies are needed to characterise these effects.

Establishment of a subchronic TRV

- Choice of the critical effect

In rodent studies, the most sensitive effects (occurring at the lowest tested doses) from subchronic oral exposure to CYN are observed in the liver and kidneys. An increase (absolute and/or relative) in the weight of these two organs has been observed several times (Humpage and Falconer, 2002; Sukenik *et al.*, 2006; Chernoff *et al.*, 2018) and has been correlated with an increase in some biochemical parameters and histological changes. For the liver, an increase in transaminases (AST and ALT) and alkaline phosphatases has been observed, as well as histological alterations (pigmentation anomalies, alterations and hypertrophy of hepatocytes, inflammation, hepatic cytolysis). In the kidneys, morphological and histological alterations occur in the tubules and the medullary zone. The increase in the weight of these organs is therefore not the result of adaption but seems to be degenerative.
The CES therefore considers the increase in organ weight (liver and kidneys) as the critical effect to be considered for establishing a TRV.

- Analysis of the existing subchronic TRVs

The CES considers that none of the current TRVs (Humpage and Falconer, 2002; AFSSA-AFSSET, 2006; US EPA, 2015) should be selected given the limitations identified in the key study used to establish these TRVs. In the study by Humpage and Falconer (2002), male Swiss Albino mice were exposed to 0, 30, 60,120 or 240 $\mu g.kg^{-1}.d^{-1}$ CYN by daily gavage for 11 weeks. The authors set a No Observed Adverse Effect Level (NOAEL) of 30 $\mu g.kg^{-1}.d^{-1}$ based on the increase in kidney weight in animals observed at the highest dose.
This study was not selected for the following reasons:

- the administered dose of the toxin is uncertain due to the lack of information on its purity and dosage during the study;
- the nature of the critical effect selected is questionable insofar as the observed increase in kidney weight can only be linked to a real pathological alteration only from the highest dose in the study;
- the authors did not conduct their study according to the OECD guidelines for a subchronic oral toxicity test (OECD, 2018) due to lack of equipment.

The CES therefore does not retain the existing values and proposes to establish a new subchronic oral TRV based on a better-quality study.

- Choice of the key study

The CES experts selected the study by Chernoff *et al.* (2018) as the key study. This 90-day study evaluated the subchronic oral effects of CYN in male and female CD-1 mice fed daily by gavage at doses of 0; 75; 150 or 300 µg.kg^{-1}.d^{-1} of CYN at more than 95% purity. A Lowest Observed Adverse Effect Level (LOAEL) of 75 µg.kg^{-1}.d^{-1} was established on the basis of the increase in liver and kidney weight observed in males.

- Choice of the critical dose

The study by Chernoff *et al.* (2018) identified a dose-response relationship between increased relative liver weight in males and exposure to CYN.
This was modelled with the software proposed by EFSA (EFSA, 2017), which uses the PROAST software package (PROAST version 65.7) developed by the Netherlands National Institute for Public Health and the Environment (RIVM) to establish a Benchmark Dose (BMD).
The values obtained from this modelling could not be retained because the first exposure dose was too far from the control dose, making the model uncertain around the BMD values. In addition, these BMD values are within the linear extrapolation range of the model, making it even more uncertain.

Because of the experimental protocol followed, the study by Chernoff *et al.* (2018) cannot be used to set a NOAEL.
The critical dose chosen is therefore the lowest dose tested, which makes it possible to fix a LOAEL based on the increase in the weight of the organs (liver and kidneys).

LOAEL = 75 µg.kg^{-1}.d^{-1}

- Allometric adjustment

An allometric adjustment was performed to reduce the value of the uncertainty regarding interspecies variability. A Human Equivalent Dose (HED) was calculated, using the following equation[1]:

$$\text{Human Equivalent Dose} = \text{Animal Dose} \times \left(\frac{\text{Animal weight}}{\text{Human weight}}\right)^{1/4}$$

In the absence of average mouse weight data for the study, an average weight of 25 g was used, as recommended by the US EPA (US EPA, 2006). The average human weight used for the calculation was 70 kg.
This corresponds to a critical dose of:

LOAEL $_{HED}$ = 10.31 µg.kg^{-1}.d^{-1}

- Choice of uncertainty factors

The TRV was calculated from the LOAEL$_{HED}$ using the following uncertainty factors, leading to an **overall uncertainty factor of 75:**

[1] This equation is taken from the recommendations of the US EPA (US EPA, 2006).

- Inter-species variability (UF_A): 2.5. The allometric adjustment performed enabled a human equivalent dose to be calculated, using the previous equation. To account for toxicodynamic variability and residual uncertainties, an additional uncertainty factor was set at 2.5 according to IPCS recommendations (IPCS, 2005) and based on ANSES practices.
- Inter-individual variability (UF_H): 10. Because there were no scientific data available to reduce the default value, the value of 10 was used.
- Use of a LOAEL (UF_L): 3. ECHA recommends using a factor of 3 when the LOAEL of the study represents the lowest dose at which the effect is observed (ECHA, 2012).
- UF_D: 1. Although the body of data is quantitatively small, the available studies are consistent and the critical effect is reproducible.

- Proposed chronic TRV and confidence level

$$\mathbf{TRV = LOAEL_{HED} / UF = 0.14\ \mu g.kg^{-1}.d^{-1}}$$

- Confidence level

The overall confidence level **moderate** was assigned to this TRV based on the following four criteria: nature and quality of the data (low), choice of the critical effect and the mode of action (high), choice of the key study (high) and choice of the critical dose (low/moderate).

4. AGENCY CONCLUSIONS AND RECOMMENDATIONS

The French Agency for Food, Environmental and Occupational Health & Safety endorses the conclusions and recommendations of the CES on "Health Reference Values" on the proposed subchronic toxicity reference value by the oral route for cylindrospermopsin (see Table 1).

As a reminder, when dealing with TRVs and in line with the scenarios generally taken into account when assessing health risks in humans, ANSES distinguishes between three types of exposure duration:

- acute exposure, from 1 to 14 days;
- subchronic exposure, from 15 to 364 days;
- chronic exposure, for 365 or more days.

Table 1: Subchronic oral TRV for cylindrospermopsin

Critical effect (key study)	Critical concentration	UF	TRV
Increased weight of liver and kidneys Chernoff *et al.* (2018)	**LOAEL = 75 µg.kg^{-1}.d^{-1}** Allometric adjustment **LOAEL$_{HED}$ = 10.31 µg.kg^{-1}.d^{-1}**	75 UF_A = 2.5 UF_H = 10 UF_L = 3	**0.14 µg.kg^{-1}.d^{-1}** **Confidence level Moderate**

The proposed subchronic oral TRV is associated with a moderate confidence rating. This is a transient TRV that may be re-evaluated if new subchronic oral studies are published that would increase knowledge of the toxicity of cylindrospermopsin by ingestion.
Moreover, it is not currently possible to reach a decision on the potential carcinogenic effect of CYN, given the very limited data available. Further studies are needed to characterise these effects. The same is true for the potential genotoxicity of CYN.

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KEYWORDS

Valeur toxicologique de référence, VTR, cyanobactéries, cylindrospermopsine, 143545-90-8, orale, subchronique

Toxicity reference value, TRV, cyanobacteria, cylindrospermopsin, 143545-90-8, ingestion, subchronic