ANSES Opinion
Request No. 2016-SA-0297
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 TRV by the oral route for microcystin-LR (CAS No. 101043-37-2)

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 (Request No. 2016-SA-0165).

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: microcystin-LR (CAS No. 101043-37-2) [referred to as MC-LR in the following opinion] and cylindrospermopsin (CAS No. 143545-90-8). 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 MC-LR. 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 the 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.

Among cyanotoxins, MC-LR is one of the 246 variants of microcystin identified to date. It is the most commonly observed variant in water bodies and the most studied to date. A literature search carried out via the Scopus and PubMed databases identified more than 5000 articles relating to MC-LR published since the Agency's previous work on the theme of cyanotoxins (AFSSA-AFSSET, 2006). After a first selection of some 40 studies considered robust by the experts, 15 studies of good scientific quality were chosen to establish a dose-response relationship for the substance in question. One of them 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 used for recreational aquatic activities. This Opinion therefore proposes a subchronic oral TRV related to the ingestion of microcystin-LR (MC-LR).

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. Three rapporteurs were appointed, the first to assess toxicological studies on the genotoxic status of MC-LR, the second to assess studies on the neurotoxicity of MC-LR, and the third to assess studies on the reproductive and developmental effects of MC-LR. 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 toxicological data on MC-LR available to date are from animal studies.

- Toxicokinetics

MC-LR is absorbed by diffusion facilitated by receptors of the organic anion transport polypeptide (OATP) class (Fischer *et al.*, 2010). After oral exposure, absorption of MC-LR was observed mainly in the stomach and small intestine (Ito and Nagai, 1997, 2000).

Distribution of the toxin to other target organs and tissues was also observed via OATPs (Komatsu *et al.*, 2007; Jasionek *et al.*, 2010; Feurstein *et al.*, 2010). After ingestion by rodents, MC-LR was found in the liver, lungs, kidneys (Nishiwaki *et al.*, 1994; Ito and Nagai, 1997, 2000) and also in brain and reproductive tissues (Li *et al.*, 2012b; Wang *et al.*, 2013a).

No studies concerning the oral route were found describing the metabolism of MC-LR. The available data suggest that MC-LR conjugates first with glutathione (GSH) and then cysteine, which increases its solubility and facilitates its excretion (Kondo *et al.*, 1996; Guo *et al.*, 2015).

No studies were found containing information on the excretion of MC-LR after oral exposure. Data on excretion of MC-LR show biphasic elimination in the blood (Falconer *et al.*, 1986; Robinson *et al.*, 1991), excretion by bile (Pace *et al.*, 1991; Stotts *et al.*, 1997a and b) and by urine and faeces (Lowe *et al.*, 2012; Sedan *et al.*, 2015).

- Acute toxicity

After acute oral exposure to MC-LR, LD_{50} values of 3 to 10 $mg.kg^{-1}$ were reported in rodents (Fitzgeorges *et al.*, 1994; Yoshida *et al.*, 1997).

The effects observed in animals occur mainly in the liver: histological alterations such as necrosis of the centrolobular region, vacuolation and steatosis (Yoshida *et al.*, 1997; Ito *et al.*, 1997a; Fawell *et al.*, 1999; Heinze *et al.*, 1999; Sedan *et al.*, 2015).

- Irritation

A single study showed irritant and sensitising effects in guinea pigs and rabbits, respectively, following dermal exposure to MC-LR (Torokne *et al.*, 2001). Sensitisation was considered moderate to severe but no correlation between the intensity of the sensitisation reaction and the concentration of MC-LR was found. All samples induced negligible to mild eye and skin irritation in rabbits. On the basis of a single study, it is not possible to conclude as to the effects of MC-LR on irritation and sensitisation.

- Subchronic and chronic toxicity

In animals, subchronic and chronic oral exposure to MC-LR induced:

- Hepatic effects: hepatomegaly, chronic inflammation, degeneration of hepatocytes (Ito *et al.*, 1997b; Fawell *et al.*, 1999; Zhang *et al.*, 2012; He *et al.*, 2017);

- Lung effects: increase in alveolar septal thickness, rupture of cell junction integrity (Li *et al.*, 2016; Wang *et al.*, 2016);
- Changes in the serum metabolic and biochemical profile: elevated transaminases, decreased total protein levels (Fawell *et al.*, 1999; He *et al.*, 2017);
- Effects on the nervous system: impaired cognitive function, histological lesions, oxidative damage, neuro-inflammation (Li *et al.*, 2012b; Li *et al.*, 2014a and b; Li *et al.*, 2015c).

- Reprotoxicity and effects on development

In rodents, several recent studies indicate that oral exposure to MC-LR induces:

- Altered sperm quality: decreased sperm count, teratospermia and decreased sperm motility (Chen *et al.*, 2011; Chen *et al.*, 2017);
- A change in serum hormone levels: decreased testosterone levels, increased levels of luteinising and follicle-stimulating hormones (Chen *et al.*, 2011);
- Histological lesions in the testicles: destruction of Leydig and Sertoli cells, fibrosis, testicular atrophy (Chen et al., *2011;* Chen *et al.*, 2016b, 2017; Zhang *et al.*, 2017b);
- Changes in the ovaries: follicle atresia, decrease in the number of primordial, primary, secondary and antral follicles (Wu *et al.*, 2015).

With respect to developmental effects, some studies in animals subjected to oral exposure to MC-LR show progeny with delayed skeletal ossification, lower body weight compared to controls and impaired prostate development (Fawell *et al.*, 2014; Zhang *et al.*, 2017). An increase in perinatal mortality was also observed (Wu *et al.,* 2015).

- Genotoxicity

The mutagenicity and genotoxicity of MC-LR or of microcystic cyanobacteria extract (MCE) were assessed according to various test protocols (bacteria, cells, animals). These measure different biological parameters or "endpoints" (primary DNA alterations, gene and chromosome mutations) under very diverse experimental conditions (method, level and exposure time). The results of *in vitro* tests of primary DNA alterations, the effects of which have been considered significant by their authors, are insufficient to conclude that there is an association with any intrinsic genotoxicity of MC-LR. There is also no evidence to date of a genotoxic oxidative mode of action of MC-LR. The results of the *in vivo* comet assays, to assess primary DNA alteration, do not permit any conclusion that there is a genotoxic effect of MC-LR *in vivo*, either orally or intraperitoneally.
Following a critical analysis of all the data on the genotoxicity of microcystin-LR based on a "weight of evidence" methodology, the CES concludes that it is impossible to conclude whether microcystin-LR is genotoxic.

- Carcinogenicity

Carcinogenesis data on oral exposure to MC-LR are limited and do not allow a decision to be made on a potential carcinogenic effect. However, the International Agency for Research on Cancer (IARC) has classified MC-LR in Group 2B (possibly carcinogenic to humans) by the intraperitoneal route based on *in vivo* studies (IARC, 2010).

Subchronic TRV

- Choice of the critical effect

In vivo, oral exposure to MC-LR (drinking water or gavage) showed various effects. MC-LR was first characterised in the studies as a hepatotoxin. Many studies in recent years have also

highlighted other harmful effects: reprotoxicity, neurotoxicity, pulmonary toxicity, carcinogenicity, etc. After analysing all the studies, including those that led to the definition of NOAELs[1] and LOAELs[2] below 40 µg.kg $bw^{-1}.d^{-1}$ (lowest NOAEL chosen for hepatotoxic effects by the various international bodies for establishing TRVs), the CES chose sperm quality alteration as the critical effect. The most relevant effects on reproduction were observed in the male reproductive system. Lastly, some effects on female fertility were observed in mice and rats. The choice of this critical effect protects against all adverse effects associated with oral exposure to MC-LR at the low doses at which reprotoxicity is observed.

In view of recent studies showing effects of MC-LR on reproduction in rodents by the oral route, and the range of very low exposure doses at which the effects occur, the CES considers the alteration of sperm quality to be a relevant critical effect for humans. This alteration includes a decrease in sperm motility and sperm count and an increase in the number of sperm abnormalities.

- Analysis of the existing subchronic TRVs

The various subchronic TRVs proposed to date are based on three studies (Fawell *et al.*, 1994; Heinze *et al.*, 1999; Kuiper-Goodman *et al.*, 1999). They cite adverse effects observed in the kidneys and propose LOAELs/NOAELs greater than 40 µg.kg $bw^{-1}.d^{-1}$. In view of the number of oral toxicity studies (via drinking water or gavage) in rodents published in recent years showing the occurrence of adverse effects (including hepatotoxicity, reprotoxicity and neurotoxicity) at exposure doses equal to or lower than the current NOAEL of 40 µg.kg $bw^{-1}.d^{-1}$, it appears necessary to establish a more protective subchronic TRV to cover all other recently demonstrated effects of subchronic oral exposure to low doses of MC-LR.

Given these observations, the CES did not retain the existing values and proposes establishing a more protective subchronic TRV.

- Establishment of a subchronic TRV
  - Choice of the key study

Five studies (Chen *et al.*, 2011; Chen *et al.*, 2015; Wu *et al.*, 2015; Chen *et al.*, 2017; Zhang *et al.*, 2017b) are of good quality, and some are ranked 1 on the Klimisch score.

Of all the studies, the CES decided to select as the key study the one by Chen *et al.* (2011) in view of the quality of the study (Klimisch score of 1), the range of relatively low doses studied (0; 1; 3.2 and 10 $\mu g.L^{-1}$), and the existence of a dose-response relationship for several reprotoxic effects (sperm quality, increased LH/FSH levels and decreased testosterone levels). It is also the study in which reprotoxic effects occurred at the lowest dose (3.2 $\mu g.L^{-1}$ or 0.48 µg.kg $bw^{-1}.d^{-1}$).

  - Choice of the critical dose

The methodological recommendations suggest that preference should be given to the establishment of a Benchmark Dose (BMD) whenever available data allow, to select a NOAEL as the second choice and a LOAEL as the last option (ANSES, 2017).

A BMD was modelled based on a dose-response relationship between decreased sperm motility and daily exposure to MC-LR for 3 months (see Appendix 5). Nevertheless, the BMD/BMDL ratio is greater than 10 and the BMD obtained is 14 times lower than the first experimental dose tested. According to the recommendations of the BMDS Wizard software developed by ICF International (ICF BMDS Wizard), a BMD/BMDL ratio greater than 5 and a BMD more than three times smaller

[1] NOAEL: No Observed Adverse Effect Level

[2] LOAEL: Lowest Observed Adverse Effect Level

than the first experimental dose tested suggest that the modelling obtained is not robust. It was therefore decided not to use this BMDL and to retain the NOAEL.

NOAEL = 1 µg.L^{-1}

This NOAEL corresponds to a concentration of MC-LR in water. A conversion factor of 0.15 recommended by EFSA (EFSA, 2012)[3] was used by default to obtain a daily dose of subchronic exposure in mice.

NOAEL = 0.15 µg.kg bw^{-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[4]:

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

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.

$NOAEL_{HED}$ = 0.15 x (0.025 / 70)$^{1/4}$ = 0.02 µg.kg bw^{-1}.d^{-1}

- Choice of uncertainty factors

The TRV was calculated from Chen *et al.* (2011) using the following uncertainty factors (ANSES, 2017):

- Inter-species variability (UF_A): 2.5. The dose 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. As it was not known whether there is a sensitive subpopulation, a final value of 10 was chosen by default for intra-species variability.

An overall uncertainty factor of 25 was thus used to establish the TRV.

- Proposed subchronic TRV

TRV = $NOAEL_{HED}$ / UF = 0.02 / 25 = 0.0008 µg.kg bw^{-1}.d^{-1} ≈ 1 ng.kg bw^{-1}.d^{-1}

- Confidence level

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

[3] This factor was established by the US National Toxicology Program (NTP) from 8 toxicity studies that included drinking water consumption and body weight of the animals tested (rats or mice).

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

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 toxicity reference values by the oral route for microcystin-LR.

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.

For the subchronic TRV, the Agency proposes a value of 1 ng.kg $bw^{-1}.d^{-1}$ (see Table 1).

Table 1: Subchronic oral TRV for microcystin-LR

Critical effect (key study)	Critical concentration	UF	TRV
Alteration in sperm quality Chen *et al.* (2011)	NOAEL = 1 µg.L^{-1} = 0.15 µg.kg $bw^{-1}.d^{-1}$) <u>Allometric adjustment</u> $NOAEL_{HED}$ = 0.02 µg.kg $bw^{-1}.d^{-1}$	25 UF_A: 2.5 UF_D: 10	**1 ng.kg $bw^{-1}.d^{-1}$** **Confidence level Moderate/high**

The Agency reiterates that, based on current knowledge, it is not possible to conclude on the genotoxicity or the carcinogenicity of MC-LR.

Lastly, the Agency notes that the TRV determined refers only to MC-LR. The Agency therefore emphasises the need to acquire knowledge of the potential toxicity of other variants of microcystin, as they may have toxic properties equivalent to or greater than those of MC-LR (identification of new toxins, performance of subchronic and chronic toxicity tests, performance of carcinogenicity assessment tests, etc.).

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KEYWORDS

Valeur toxicologique de référence, VTR, microcystine-LR, 101043-37-2, exposition orale, subchronique, reprotoxicité

Toxicity reference value, TRV, microcystin-LR, 101043-37-2, oral exposure, subchronic, chronic, reprotoxicity