ANSES Opinion

Request No 2016-SA-0299

Related Requests: Nos 2016-SA-0165, 2015-SA-207 and 2016-SA-0298

Maisons-Alfort, 16 March 2020

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

on the proposed acute oral TRV for saxitoxin (CAS No. 35523-89-8)

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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 16 March 2020 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 drinking water, 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 drinking water, and water for bathing and other recreational activities, it became necessary to update the body of reference work concerning the toxicity of cyanotoxins.

Three 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 three toxins: saxitoxin (CAS No. 35523-89-8) [referred to as STX in the following opinion], microcystin-LR (CAS No. 101043-37-2), and cylindrospermopsin (CAS No. 143545-90-8). The Agency is publishing the results of this work in three separate opinions (one for each toxin).

The purpose of this opinion is therefore to propose an acute oral TRV for STX. This TRV will be used in particular 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 hypothesis is to consider a monotonic relationship between exposure (dose) and effect (response). 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 a dose-response relationship to be established;
- 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 structured and rigorous approach involving collective assessments by groups of specialists.

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 appraisal was carried out by the Expert Committee on "Health reference values" (HRV CES). The methodological and scientific aspects of the work were presented to the CES. Two rapporteurs were appointed to evaluate toxicological studies eligible for the establishment of an acute oral TRV for STX. The work was adopted by the HRV CES at its meeting on 17 October 2019.

ANSES analyses interests declared by experts before they are appointed and throughout their work in order to prevent risks of conflicts of interest in relation to the points addressed in expert appraisals. The experts' declarations of interests are published on the ANSES website (www.anses.fr).

3. ANALYSIS AND CONCLUSIONS OF THE CES

Summary of the toxicological data

The summary of toxicological data was written based on the report of the European Food Safety Authority (EFSA) published in 2009 (EFSA, 2009), supplemented by a literature search undertaken between 26 November and 17 December 2018, whose query criteria are given in Annex 2 of the report associated with this opinion.

- Toxicokinetics

After a single intravenous (i.v) injection in rats, STX is rapidly distributed in the bloodstream and then in the rest of the body (Garcia *et al.,* 2004; Andrinolo *et al.*, 1999). When administered orally, STX crosses the intestinal barrier and enters the general bloodstream despite the first-pass effect through the liver, giving it access to the peripheral nervous system (PNS), which is its main target (Andrinolo *et al.,* 2002; Torres *et al.,* 2007).

In humans, STX and its analogues, such as neosaxitoxin (neoSTX) and gonyautoxins (GTX), are also distributed in numerous organs, tissues and biological fluids (brain, bile, cerebrospinal fluid, liver, spleen, heart, thyroid, adrenal glands, kidneys, pancreas and lungs), regardless of the exposure route.

Moreover, study results have shown that STX and its analogues initially found in gastric contents undergo metabolic transformations for three to four hours following poisoning in humans (Garcia *et al.,* 2004; Llewellyn *et al.,* 2002).

Glucuronides resulting from oxidation (Phase I) and glucuronidation (Phase II) reactions are often the metabolic end products. They are eliminated mainly via urine and, to a lesser extent, through bile (Garcia *et al.,* 2010; Testai *et al.,* 2016). The efficient excretion of STX also depends on its affinity for the various voltage-gated sodium channel (Na^+) sub-types, called Na_v.

Other studies have corroborated these results, in particular those showing that gonyautoxins 2 and 3 (GTX-2 and -3) are primarily excreted by glomerular filtration in humans and animals (Andrinolo *et al.,* 1999; Andrinolo *et al.,* 2002). Recently, a case of paralytic shellfish poisoning (PSP) in Alaska demonstrated the renal excretion of STX and of other paralytic shellfish toxins (PSTs) when the patient's urine was analysed by high-performance liquid chromatography (HPLC) (Coleman *et al.,* 2018).

Depending on the study, species and biological matrix, the elimination half-life of STX ranges from 1.5 to 10 hours, which is consistent with the short duration of the symptoms observed following poisoning (Kao, 1993; Andrinolo *et al.,* 1999; Gessner *et al.,* 1997).

Regarding potential faecal excretion, experimental studies show that this route appears to be very uncommon or even non-existent (Hines *et al.,* 1993; Stafford and Hines, 1995; Andrinolo *et al.,* 1999).

- Acute and subacute toxicity

The majority of cases of human poisoning involve neurological and muscular symptoms. More specifically, early signs of poisoning such as paraesthesia with prickling, tingling or numbness around the mouth and in the extremities, which appear rapidly, can be followed by muscular paresis

and paralysis that may be life-threatening if the respiratory muscles are involved (Arnich and Thébault, 2018).

Other symptoms, such as headaches, hypersalivation, intense thirst, sweating, vomiting, diarrhoea and digestive pain, can also occur in poisoned individuals (Rapala *et al.,* 2005). After 12 hours, when respiratory impairment has not led to the death of the patient, the outcome is favourable within a few hours to a few days. There is currently no specific treatment: management is symptom-based and initiated as needed.

The most recent meta-analysis of cases of human poisoning established a critical minimal dose of 0.37 µg STX eq·kg^{-1} bw, corresponding to a probability higher than 10% of showing symptoms (Arnich and Thébault, 2018). This critical dose selected by the authors of this meta-analysis represents a dose around four times lower than the LOAEL of 1.5 µg STX eq·kg^{-1} bw established by EFSA, based on a set of symptoms described as “mild” (EFSA, 2009).

The majority of the available experimental studies were undertaken by exposing animals via several different routes, including drinking water, gavage, and the i.v and intraperitoneal (i.p) routes. These acute toxicity studies mainly describe median lethal doses (LD_{50}), with no attempt by the authors to characterise a dose-effect relationship, because the toxicity mechanism of STX and its derivatives makes the establishment of such a relationship extremely difficult.

The CES identified the following three experimental studies undertaken in mice as being the most informative with regard to the toxicity of STX and its analogues in animals: those by Munday *et al.* (2013), Selwood *et al.* (2017) and Finch *et al.* (2018). They all used the same study protocol and were undertaken by the same teams of researchers. These three studies’ results supported one another, as they were substantially similar.

The study by Munday *et al.* (2013) found that the acute toxicity of all of STX’s analogues by gavage was significantly lower than by i.p injection, and that acute toxicity via feed was lower than that by gavage. Moreover, neoSTX was reported as being significantly more toxic than STX, by gavage and via feed. The CES underlines that the toxic equivalency factors (TEFs) for STX analogues have widely varying values in the scientific literature. The study by Selwood *et al.* (2017) also suggested revising the values defined by EFSA in 2009.

The most suitable indicators for the sublethal effects mentioned in the study by Munday *et al.* (2013) were abdominal respiration, lethargy, and a decrease in exploratory behaviour in mice. The study reported that a reduction in grip strength was only observed at doses slightly below the LD_{50}.

Therefore, the experimental studies confirm the symptoms observed in cases of acute human poisoning. This consistency is due to the mechanism of action of STX and its analogues, which is the same regardless of the species exposed to the toxin.

- Irritation

Several cases of poisoning in children between the ages of two and 10 years, following the contamination of Finnish lakes by blooms of various species of STX-producing cyanobacteria, resulted in skin and eye irritation. The authors attributed these toxic effects, associated with systemic signs such as abdominal pain and fever, to STX and its analogues (Rapala *et al.,* 2005).

The skin toxicity caused by the cyanobacterial contamination of recreational water has been attributed to lipopolysaccharides (LPSs), components of the cellular walls of all cyanobacteria. LPSs, found in all Gram-negative bacteria, can cause irritative and allergic reactions in human and animal tissues (Pilotto *et al.,* 2004). Other non-STX-producing species of cyanobacteria can therefore induce skin irritation.

- Sensitisation

In general, the sensitivity of individuals to cyanobacteria in bathing water varies widely, because there can be allergic reactions to cyanobacteria as well as reactions directly induced by toxins. For example, cyanobacterial pigments can be responsible for allergic reactions (Cohen and Reif, 1953).

Cyanobacteria share some characteristics with common airborne allergens, and studies have shown allergic responses to cyanobacteria in patients with allergic rhinitis and/or asthma. A recent detailed case study reported an allergic reaction characterised by severe facial swelling with periorbital oedema and an erythematous pruritic skin rash on the patient's arms and hands after she had gone swimming in a lake. The analysis of a sample of the patient's serum showed an increase in immunoglobulin E (IgE) specific to the cyanobacterial extract, after excluding other underlying causes (Geh *et al.,* 2016).

- Subchronic and chronic toxicity

The majority of the experimental studies on subchronic/chronic toxicity have demonstrated the neurotoxicity induced by STX and its analogues. This neurotoxicity takes the form of:

- changes in total antioxidant capacity (TAC) and the production of reactive oxygen species (ROS) in the brain, especially the hippocampus, and in the liver, associated with a reduction in antioxidant defences (Ramos *et al.,* 2014);
- a decrease in the activity of glutamate cysteine ligase (GCL) as well as an increase in concentrations of glutathione and glutathione S-transferase (GST) in the brain (Ianora *et al.,* 2011);
- a reduction in aversive and spatial memory performance, demonstrate by inhibitory avoidance (IA) and Morris water maze (MWM) tests;
- an increase in levels of amino acid neurotransmitters (aspartate, glutamate, GABA) in the brain (Cianca *et al.,* 2009);
- acute alteration of the production of dopamine (DA) and its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC) (Cianca *et al.,* 2011);

STX therefore disrupts the homeostasis of certain central neurotransmission pathways. Moreover, given that neuronal excitability plays a major role in the normal development of the central nervous system (CNS), prolonged exposure to PSTs can affect neurogenesis (Brackenbury *et al.,* 2010).

Furthermore, a transient reduction in body weight and feed intake after repeated administration (12 weeks), as well as transient cholestasis at the end of the treatment period, were observed in Sprague-Dawley rats exposed subcutaneously to 6 $\mu g \cdot kg^{-1}$ of neoSTX (Zepeda *et al.,* 2014).

- Reprotoxicity and effects on development

No studies on potential reproductive or developmental toxicity in humans or animals are currently available for SXT or its analogues. However, two studies evaluated the impact of STX on the development of zebrafish: exposing zebrafish larvae to STX (229 µg STX eq$\cdot L^{-1}$) for seven days induced a reduction in sensory-motor functions within 48 hours and paralysis after four days. At twice the dose (481 µg STX eq$\cdot L^{-1}$), the toxin caused oedema in several organs (eyes, pericardium, yolk sac). Lastly, larval paralysis, whose intensity depended on the stage of development, was observed. The study noted that these sublethal morphological and sensory-motor effects were reversible when these larvae were then placed in water free of any toxins. Zebrafish temporarily exposed to STX

during larval development (two to four days after fertilisation) had reduced growth and survival (Lefebvre *et al.*, 2004). However, the concentrations used in these studies were higher than those to which individuals are likely to be exposed over a long period (Oberemm *et al.,* 1999; Lefebvre *et al.,* 2004). It is therefore difficult to extrapolate these experimental data to humans.

- Genotoxicity

There are very few studies on the potential genotoxicity of STX and its analogues. A recent study, by Melegari *et al.* (2015), did not demonstrate any significant change in the frequency of binucleated cells with micronuclei (MN) in N2A and Vero cells exposed to STX, indicating a lack of genotoxicity in these study conditions.

Conversely, genotoxic effects on *Hoplias malabaricus* freshwater fish were observed (Da Silva *et al.,* 2011). Another study by Da Silva *et al.* (2014) confirmed these results by exposing *H. malabaricus* neuronal cells to a concentration of 3 $\mu g \cdot L^{-1}$ STX eq. During this exposure, the comet assay showed an increase in DNA damage (Da Silva *et al.,* 2014).

- Carcinogenicity

The International Agency for Research on Cancer (IARC) has not studied STX with regard to its carcinogenicity. According to the CES, this is due to a profound lack of data in the scientific literature, making it impossible to determine the carcinogenic potential of STX.

- At-risk populations

According to a case study involving an outbreak of mollusc (*Amphichaena kindermani*) poisoning between July and August 1987, on the Pacific Coast of Guatemala, children appear to be the most susceptible population group. Of the 187 poisoned individuals in this study who showed symptoms, children under the age of six had a higher mortality rate (50%) than people over the age of 18 (7%) (Rodrigue *et al.*, 1990).

Acute TRV

- Choice of the critical effect

As seen above, the first effects that occur at the lowest doses in humans after poisoning with STX or its analogues are peri-oral paraesthesia, light-headedness, headaches, nausea, vomiting and dizziness. These symptoms are followed by asthenia, muscle weakness and unintelligible speech. In poisoned individuals, these symptoms rapidly progress to muscle paralysis and severe respiratory difficulty that can lead to death (Arnich and Thébault, 2018).

Binding to Na_v channels and the blockage of their conductance are considered as the major molecular mechanism of neurotoxicity for STX and its analogues (EFSA, 2009).

In experimental studies, cases of animal poisoning are also characterised by symptoms with increasing severity. The quantitative evaluation, at sublethal doses, of the reduction in grip strength in the study by Munday *et al.* (2013) supplemented the qualitative observations of symptoms in animals following exposure to STX. However, the choice of a critical effect is difficult, because signs

of benign neurological dysfunctions can rapidly progress to serious disorders potentially leading to death.

Due to the uncertainties reported in the epidemiological studies estimating the exposure of poisoned individuals, the CES proposes not selecting the paraesthesia observed in humans, in certain cases of non-benign poisoning, as the critical effect.

The CES proposes instead selecting the skeletal muscle dysfunction generated by the blockage of Na_v channels, observed in experimental studies, as the critical effect. Moreover, it draws attention to the difficulty of establishing a dose-effect relationship for the toxicity of STX and its analogues, due to their toxicity mechanism.

- Analysis of the existing acute TRVs

Given the limitations of the epidemiological studies, related in particular to the difficulty of precisely characterising the exposure of poisoned individuals, the CES decided to not use the available epidemiological studies for the establishment of a TRV, or the acute reference dose of 0.5 µg STX eq·kg bw^{-1}·day^{-1} established by EFSA in 2009.

Thus, the CES proposes establishing a new acute oral TRV based on an experimental study.

- Establishment of an acute TRV

    - Choice of the key study

Since the epidemiological studies include various biases, the CES proposed determining the TRV based on one of the available experimental studies. Three experimental studies were identified by the CES, because they are the most recent studies suitable for the establishment of a TRV. They were the studies by Munday *et al.* (2013), Selwood *et al.* (2017) and Finch *et al.* (2018), mentioned above.

There were few differences between these studies, undertaken by the same team of researchers. Nonetheless, **the study by Munday *et al.* (2013) was selected as the key study** because it alone supplemented the investigation of the toxic effects of STX via the grip strength test, which confirmed the results of the 14-day visual observation conducted in the three studies.

Moreover, the study by Selwood *et al.* (2017), whose objective was to determine the acute toxicity of STX's analogues to compare it with that of STX, used the NOAEL from the study by Munday *et al.* (2013) of 544 nmol·kg^{-1} by gavage in its table of results. This motivated the CES to directly use the source study that determined this NOAEL.

    - Choice of the critical dose

The CES stresses the difficulty of determining a critical dose due to the lack of a clear dose-effect relationship in the selected key study, and therefore the difficulty of modelling a benchmark dose (BMD). Despite the lack of an observable dose-effect relationship in this study and the low level of detail concerning the symptoms occurring before death, the NOAEL of 544 nmol·kg^{-1} could be considered as a starting point for the establishment of a TRV. However, the CES draws attention to the very narrow dose interval between the observed NOAEL and the LD_{50} values described in the study. Given this narrow interval, the CES warns against the severity of possible poisoning if the established TRV is exceeded.

The CES underlines that to make up for the lack of information on the interval between the NOAEL and the LOAEL in the key study selected, it would make sense to undertake a study multiplying exposure doses over a close range, to be able to better characterise a dose-effect relationship. A robust study, undertaken via a representative route of administration such as exposure by drinking water, could enable the slope corresponding to the dose-effect relationship to be modelled and its intensity to be assessed. In light of the difficulty of quantifying the observed effects, the said study should use the most appropriate neurotoxicity tests.

The CES therefore considered the NOAEL described in the study by Munday *et al.* (2013) as the threshold below which Na_v channel blockage is not expected to be sufficient to induce observable muscular paralysis in exposed animals.

NOAEL = 544 nmol·kg^{-1} = 163 µg·kg^{-1}

- Allometric adjustment

An allometric adjustment was performed to reduce the value of the uncertainty regarding inter-species variability. A human equivalent dose (HED) was calculated, using the following equation[1]:

$$\text{Dose équivalent e Homme} = \text{Dose animal} \times \left(\frac{\text{Poids animal}}{\text{Poids homme}} \right)^{1/4}$$

Dose équivalente Homme	Human equivalent dose
Dose animal	Animal dose
Poids animal	Animal body weight
Poids homme	Human body weight

A body weight for mice of 22 g was used for the calculation, based on the body weight range for mice indicated in the study (18 – 22 g), to be better aligned with the recommendation of the US EPA (US EPA, 2006) of 25 g. For humans, the body weight used for the calculation was 70 kg.

$NOAEL_{HED}$ = 163 x (0.022 / 70)$^{1/4}$ = 22 µg·kg^{-1} bw

- Choice of uncertainty factors

Based on the study by Munday *et al.* (2013), the TRV was calculated using the following uncertainty factors (ANSES, 2017):

- 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.

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

- Inter-individual variability (UF_H): 10. The observed human data, according to the study by Rodrigue *et al*. (1990), suggest that children are a susceptible population group. This led the CES to choose a default value of 10 for inter-individual variability.
- Inadequacy of the data (UF_D): 10. The epidemiological and experimental studies include various biases. These are combined with other shortcomings such as a lack of available information regarding the toxicity of each of STX's analogues. Moreover, due to the severity of the selected critical effect and to mitigate potential risks associated with other analogues such as neoSTX, the CES chose to apply a UF_D of 10.

An overall uncertainty factor of 250 was thus used to determine the TRV.

- Proposed acute oral TRV

$TRV = NOAEL_{HED} / UF = 22 / 250 = 0.088\ \mu g \cdot kg^{-1}\ bw \cdot day^{-1} \approx 0.1\ \mu g \cdot kg^{-1}\ bw \cdot day^{-1}$

- Confidence level

The overall confidence level **low** 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 (moderate-low) and choice of the critical dose (low). This confidence level may be reassessed subject to new studies enhancing the body of data on the toxicity of STX.

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” regarding a proposed acute oral TRV for STX.

As a reminder, as part of the scenarios generally taken into account when assessing human health risks, when TRVs are used, ANSES distinguishes between three types of exposure durations:

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

An acute oral TRV is being proposed for STX, based on skeletal muscle dysfunction. A **low** confidence level was assigned to this TRV.

Table 5: Acute oral TRV for STX

Critical effect (key study)	Critical concentration	UF	TRV
Skeletal muscle dysfunction Munday *et al.,* 2013	NOAEL = 163 $\mu g \cdot kg^{-1}\ bw \cdot day^{-1}$ <u>Allometric adjustment</u> $NOAEL_{HED}$ = 22 $\mu g \cdot kg^{-1}\ bw \cdot day^{-1}$	250 UF_A: 2.5 UF_D: 10 UF_H: 10 UF_L: 1 UF_S: 1	**TRV = 0.1 $\mu g \cdot kg^{-1}\ bw \cdot day^{-1}$** **Confidence level** **Low**

The Agency reiterates the narrow interval between the doses leading to the first observable harmful effects and the death of individuals. It fully supports the CES's recommendation to remedy the lack of knowledge on this interval by undertaking a study multiplying exposure doses over a narrow range.

This study, conducted by the oral route using the HPLC method, should enable the slope corresponding to the dose-effect relationship to be modelled and its intensity to be assessed. In light of the difficulty of quantifying the observed effects, the study should use the most appropriate neurotoxicity tests. It should also characterise the toxicity of STX analogues, found in non-negligible quantities during cases of human poisoning.

The Agency stresses that this TRV is likely to be reassessed if such a study is published and enhances knowledge on the toxicity associated with the ingestion of STX and its analogues. Despite the low confidence level defined for this TRV, the Agency reiterates that given the high toxicity of STX and the public health challenges posed by cyanotoxins for French regional health agencies, the CES decided to propose a TRV for STX, which represents a first step in protecting the French population from potential exposure to STX. Lastly, the Agency highlights the severity of possible poisoning if the proposed TRV is exceeded.

Dr Roger Genet

KEYWORDS

Toxicity reference value, TRV, cyanobacteria, saxitoxin, acute, neurotoxicity

Mots – clés

Valeur toxicologique de référence, VTR, cyanobactéries, saxitoxine, aiguë, neurotoxicité