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

FRONT OFFICE FOOD AND PRODUCT SAFETY

COMMENTS ON THE EFSA OPINION ON TETRODOTOXIN (TTX) AND TTX-ANALOGUES IN MARINE BIVALVES AND GASTROPODS

Risk assessment requested by:	NVWA-BuRO
Risk assessment performed by:	RIVM[1]
Date of request:	20-04-2017
Date of risk assessment:	12-05-2017 (first draft) 19-05-2017 (second draft) 24-05-2017 (final)

Subject

Recently, TTX and some of its analogues have been detected in gastropods and bivalves from European waters. Early 2017, the Institute for Risk Assessment Sciences of Utrecht University (IRAS) has published results of *in vitro* research on the neurotoxicity of TTX (Kasteel and Westerink, 2017). On 20 April 2017, the Panel on Contaminants in the Food Chain (CONTAM Panel) of the European Food Safety Authority (EFSA)[2] published an opinion[3] on TTX and TTX-analogues in marine bivalves and gastropods (EFSA, 2017a).

Questions

NVWA-BuRO would like to have answers to the following questions:

1. Is there sufficient scientific basis for the health based guidance value (HBGV) that has been derived in the EFSA Opinion on TTX?
2. Is there sufficient scientific basis for the concentration TTX in shellfish meat that is not expected to lead to adverse effects in humans (critical concentration, CC) that has been identified in the EFSA Opinion on TTX?
3. Have the results from the IRAS study (Kasteel and Westerink, 2017) (sufficiently) been taken into account in the EFSA Opinion? If not, could the results give cause for a change in the HBGV or the CC?

[1] The scientific validity of this assessment has been assessed and approved by an expert from RIKILT Wageningen UR

[2] Referred to as 'EFSA CONTAM Panel' in the remainder of the document.

[3] Referred to as 'EFSA Opinion' in the remainder of the document

Conclusions

Answer to questions 1 and 2:
FO considers the available (limited) human data in combination with the reference point of 25 µg/kg bw as selected by the EFSA CONTAM Panel from the Abal *et al.* (2017) study an insufficient scientific basis to derive an ARfD for TTX. The same holds for the derivation of a CC in shellfish based on the derived ARfD. FO proposes to consider these values as indicative values.

Answer to question 3:
The results from the IRAS study have not been taken into account in the EFSA Opinion, since these were not available in time for the EFSA CONTAM Panel. The IRAS study is well performed and gives insight in interspecies differences in sensitivity in inhibiting neuronal activity in rat cells and human cells *in vitro*. However, this study in combination with the other information on interspecies differences provided in the paper does not provide sufficient basis to adjust the uncertainty factor for interspecies differences used in the extrapolation from mice to humans for derivation of a HBGV.

Additional conclusions and recommendations:
FO considers the $BMDL_{10}$ for mortality derived from the Abal *et al.* (2017) study by the EFSA CONTAM Panel as the most reliable reference point to estimate an indicative reference value for risk management purposes. FO recommends to apply an extra uncertainty factor of 10 for severity of the effect (10% mortality) and possible overestimation of the true $BMDL_{10}$ in addition to the standard uncertainty factor of 100 (10 for interspecies differences and 10 for intraspecies differences). This approach gives an indicative reference value of 0.11 µg/kg bw. For a person weighing 70 kg eating 400 g of mussel or oyster meat, the shellfish meat may contain no more than 19.3 µg/kg TTX in order to keep the exposure to TTX less than or equal to 0.11 µg/kg body weight. It should be noted that, based on the above, a 20 kg child should consume no more than 115 grams of this shellfish meat.

FO notes that the indicative reference value derived on the basis of human data (0.13 µg/kg bw; FO, 2015, 2016), the indicative reference value on the basis of the Abal *et al*. (2017) study (0.11 µg/kg bw), and the ARfD derived by the EFSA CONTAM Panel (0.25 µg/kg bw) for TTX range from 0.1 – 0.25 µg/kg bw, so the differences between these derived (indicative) reference values are small, despite the different approaches in the derivation of the values.

FO recommends to perform an APROBA analysis to obtain more insight in the uncertainties in the assessment and the level of conservatism in the derived (indicative) reference value. An APROBA analysis could also indicate what type of additional information would be helpful to reduce the uncertainties in the assessment.

Introduction

TTX is a hydrophilic heat-stable toxin, produced by bacteria that can be found in certain fish species but also in marine gastropods and bivalves. TTX is a potent blocker of voltage-gated sodium channels in the nervous system and in other tissues. TTX can cause serious poisoning and death after ingestion of small doses. Altogether, 25 naturally occurring analogues of TTX have been detected and many of these have also been shown to have toxic potential (EFSA, 2017a).

In 2015, TTX was detected in mussels and oysters caught in Dutch waters. NVWA-BuRO (NVWA - Office for Risk Assessment and Research) has issued an advice in 2016 (NVWA, 2016a, 2016b). This advice was based on a risk assessment of TTX in shellfish from the Front Office Food and Product Safety (FO) (Front Office, 2015, 2016), which was performed using the information available at that point in time. Early 2017, the Institute for Risk Assessment Sciences of Utrecht University (IRAS) has published results of *in vitro* research on the neurotoxicity of TTX (Kasteel and Westerink, 2017).

On 20 April 2017, the EFSA CONTAM Panel published an opinion on TTX and TTX-analogues in marine bivalves and gastropods (EFSA, 2017a). The study by Kasteel and Westerink (2017) was, however, not part of this evaluation, since it was published after EFSA's data collection deadline. In the present assessment, only the parts of the EFSA Opinion on the derivation of the HBGV and CC are reviewed. Other parts (e.g. occurrence data, exposure assessment and analytical methods) are not considered in this assessment.

Summary of information from the EFSA Opinion and IRAS study

Description of derivation HBGV and CC as done by the EFSA CONTAM Panel

Based on the pronounced toxicity of TTX upon acute exposure, the EFSA CONTAM Panel decided to derive an Acute Reference Dose (ARfD). The use of human data, extrapolation from data on saxitoxin (STX) and the use of animal data were all considered as possible options for deriving a point of departure.

Assessment of human data by the EFSA CONTAM Panel

The EFSA CONTAM Panel noted that overall, the human case reports indicate that poisoning can result from TTX exposures in the region of 4–42 µg/kg bodyweight (bw) and higher, but these values are subject to multiple limitations (e.g. no defined amounts of actual consumption, material analysed might not represent material that has actually been consumed, concentration estimates of TTX might not be accurate due to a lack of certified standards). Moreover, the EFSA CONTAM Panel was unable to identify the original data underlying the Minimal Lethal Dose (MLD) of 2 mg/person as reported by several authors. The EFSA CONTAM Panel noted inconsistencies in the use of the term MLD in human case studies, which was used for both minimum and median lethal dose. Because of the overall limitations of the available human data, these were not used to derive the ARfD, but only as supportive information.

Assessment of extrapolation from STX by the EFSA CONTAM Panel

The EFSA CONTAM Panel noted that the mode of action of TTX is similar to that of STX, with differences in the affinity for the different subtypes of voltage-gated sodium channels. The EFSA CONTAM Panel previously established an ARfD of 0.5 µg STX equivalents/kg bw (EFSA, 2009). The database for STX is much more extensive than that for TTX. The EFSA CONTAM Panel also noted that the difference in toxic potency observed after intraperitoneal (i.p.) vs oral treatment in mice was fairly similar between STX (i.p. and oral LD_{50} values of 9.0–11.6 and 260–263 µg/kg bw respectively) and TTX (i.p. and oral LD_{50} values of 8.2–10.7 and 232–532 µg/kg bw, respectively), which according to the EFSA CONTAM Panel suggested no large differences in toxicokinetics, at least in mice. The EFSA CONTAM Panel indicated that extrapolating the ARfD for STX to TTX would result in an ARfD for TTX of 0.5 µg/kg bw.

The EFSA CONTAM Panel decided to use the data on STX only as supporting information in the derivation of a HBGV, and recommends to explore if STXs and TTXs should be combined in one health-based guidance value regarding their similar toxic effects and mode of action.

Assessment of animal data by the EFSA CONTAM Panel

The EFSA CONTAM Panel identified an oral LD_{50} study in Swiss female mice as the critical study for derivation of a reference point (Abal *et al.*, 2017). This study applied a modified 4-Level Up and Down Procedure (according to Organisation for Economic Co-operation and Development [OECD], 2008), using a certified standard of the toxin.

The Panel noted that a No-Observed-Adverse-Effect-Level (NOAEL) of 75 µg/kg bw was established by the authors of the study. The Lowest-Observed-Adverse-Effect-Level (LOAEL) was 125 µg/kg, at which apathy was observed in 9/9 animals. At the higher doses, lethality was observed, i.e. at 250 µg/kg bw (4 out of 7 animals), 500 µg/kg bw (4 out of 5 animals) and 1,000 µg/kg bw (3 out of 3 animals).

The EFSA CONTAM Panel stated that it was not possible to derive a benchmark dose (BMD) for apathy (using the recent EFSA guidance on the BMD, EFSA, 2017b). For lethality, a $BMDL_{10}$ of 112 µg/kg bw was derived ($BMDU_{10}$ = 250 µg/kg bw). The EFSA CONTAM Panel noted that this is only slightly above the NOAEL for apathy (75 µg/kg bw). Furthermore, according to the EFSA CONTAM Panel, with a group size of nine individuals at the dose of 75 µg/kg bw, it cannot be excluded that effects can occur at 75 µg/kg bw. Therefore, the EFSA CONTAM Panel selected the next lower dose tested (25 µg/kg bw) as the reference point to derive an ARfD. This dose is 4.5-fold lower than the $BMDL_{10}$ for lethality.

An ARfD of 0.25 µg/kg bw was derived applying a standard uncertainty factor (UF) of 100 to the reference point of 25 µg/kg bw. The EFSA CONTAM Panel noted that this ARfD is 16-fold lower than the dose at which severe effects have been observed in humans (4 µg/kg bw) and twofold lower than the ARfD for STX (0.5 µg/kg bw). The EFSA CONTAM Panel noted that the ARfD is a group ARfD that should apply to TTX and its analogues, taking into account their relative potencies as compared to TTX, estimated to be 0.75 for 11-oxoTTX, 0.14 for 11-deoxyTTX, 0.19/0.17 for S/R 11-norTTX-(6)-ol, 0.16 for 4-epiTTX, 0.02 for 4,9-anhydroTTX and 0.01 for 5,6,11-deoxyTTX. All these relative potencies are smaller than one, indicating lower potencies for the analogues of TTX.

The EFSA CONTAM Panel did not set a TDI for TTX and its analogues as no data on long-term effects of TTX were identified.

The EFSA CONTAM Panel made the following observations concerning the critical study and derivation of the ARfD:

- The EFSA CONTAM Panel noted the narrow interval between the dose where lethality occurred in mice (250 µg/kg bw) and the dose at which no apathy was observed (75 µg/kg bw).
- TTX was administered as a single intragastric dose in mice. The applied method excludes absorption of the compound in the very upper part of the digestive system (i.e. mouth and oesophagus), which given the rapid onset of effects, seem potential sites of TTX absorption in humans.
- The EFSA CONTAM Panel also noted that the observation period in the study (2 h) is relatively short, even for an acute toxicity study and that this precluded the observation of potential deaths and other effects occurring at later time-points, as observed by Vlamis *et al.* (2015) with shellfish extracts spiked with TTX at relatively low levels.
- The EFSA CONTAM panel noted that effects in mice occurred at oral doses more than 30-fold higher than the lowest dose reported to cause serious toxicity in humans (4 µg/kg bw) and that this suggests considerable interspecies differences between mice and humans, and between humans. As such, the EFSA CONTAM Panel concluded that the applied uncertainty factors amounting to 100, do not necessarily lead to an overestimation of the risk.
- The EFSA CONTAM Panel noted that the derivation of relative potencies for TTX analogues is associated with a high level of uncertainty since the underlying methods and data are poorly described.

Derivation of a CC in shellfish by the EFSA CONTAM Panel
The EFSA CONTAM panel concluded that, based on a large portion size (400 g), an adult body weight of 70 kg and a group ARfD of 0.25 µg/kg bw, a concentration lower than 44 µg of TTX and/or its equivalent toxic amount of its analogues per kg shellfish meat is not expected to lead to adverse effects in humans.

Summary of the IRAS study
The IRAS study (Kasteel and Westerink, 2017) has not been included in the EFSA Opinion, because the paper was not available in time for inclusion. Below, a description of the new primary data (i.e. *in vitro* test) described in this paper is given. In the IRAS study, the inhibitory effect of TTX on neuronal electrical activity has been tested in both primary rat cortical cultures and in human-induced pluripotent stem cell (hIPSC)-derived iCell® neurons in co-culture with hIPSC-derived iCell® astrocytes. This was measured by micro-electrode array (MEA). The calculated IC_{50} values for TTX amounted to 7 and 10 nM for rat and human cells, respectively. The authors concluded that TTX is roughly equipotent in both the rat and human *in vitro* models, indicating that for inhibition of neuronal activity by TTX, rat-human interspecies differences are limited. The authors also indicate that it is important to note that currently most *in vitro* models do not sufficiently take into account factors such as bioactivation/metabolism or exposure routes such as the gastrointestinal tract, but that nevertheless, these in vitro studies can shed light on the degree of interspecies variation, which can subsequently be used to further shape risk assessments based on (existing) experimental data.
The authors of the study also provide an overview of interspecies differences in *in vitro* sodium channel blocking in the discussion of their paper. In addition, the authors provide a comparison of minimum lethal doses and LD_{50} values for TTX in different species. Based on the above, the authors concluded that interspecies differences are limited for the effects of TTX.
The original papers that have been cited in the IRAS paper have not been reviewed for the current assessment.

FO comments on the EFSA Opinion and the IRAS study

FO comments on the derivation of the ARfD and CC by the EFSA CONTAM Panel

Based on the pronounced toxicity of TTX upon acute exposure, the EFSA CONTAM Panel decided to derive an Acute Reference Dose (ARfD). FO agrees with the decision of the EFSA CONTAM Panel not to derive a HBGV for chronic effects, based on the compiled knowledge.

FO comments on the assessment of human data by the EFSA CONTAM Panel
It is noted that the same human observations were available for the EFSA CONTAM Panel as for the FO assessment (Front Office, 2015, 2016). Also, the EFSA CONTAM Panel reported the same dose ranges at which toxic and lethal effects in humans are reported. The EFSA CONTAM Panel concluded that there are considerable uncertainties in the interpretation of these observations and concluded that this information should not be used for derivation of a HBGV but only as supporting evidence. FO agrees with these conclusions of the EFSA CONTAM Panel. In the previous FO assessments (2015, 2016) it was also concluded that no HBGV can be derived from these data.

FO comments on the assessment of extrapolation from STX by the EFSA CONTAM Panel
FO agrees with the EFSA CONTAM Panel's approach to use the ARfD for STX as supportive information as well as with the recommendation to explore if STXs and TTXs should be

combined in one health-based guidance value regarding their similar toxic effects and mode of action.

FO comments on the assessment of animal data by the EFSA CONTAM Panel
FO agrees that the animal study performed by Abal *et al.* (2017) provides a better basis for risk assessment than only the human data. This study was not available at the time of the earlier FO evaluations (Front Office, 2015, 2016). FO agrees with the derivation of the $BMDL_{10}$ of 112 µg/kg bw for lethality from this study. The EFSA CONTAM Panel noted that this is only slightly above the NOAEL for apathy derived by Abal *et al.* (75 µg/kg bw).

FO agrees with the EFSA CONTAM Panel that in the Abal *et al.* (2017) study, apathy and mortality are observed at doses which are close to each other and that the dose response curve of TTX is steep.

In addition, FO notes in line with the EFSA CONTAM Panel that given the short observation period in the study (2 h), it is not known whether the animals with apathy would recover or would die, had the observation period been longer. The observed apathy may be a reversible sublethal effect which occurs at lower doses than and in the absence of mortality, but also an effect that precedes mortality in time. It is unclear from this study which of these options is applicable for the observed apathy.

Additionally, the EFSA CONTAM Panel indicated that a BMDL cannot be derived for apathy using the method recommended as optimal in the recent EFSA guidance on the BMD (EFSA, 2017b). This method is called "model averaging" [4]. However, the guidance also mentions that the BMDL can be derived based on the single models, in cases where model averaging is not possible. FO applied this surrogate method and estimated a $BMDL_{10}$ for apathy of 70 µg/kg bw (with $BMDU_{10}$ = 125 µg/kg bw, see Appendix I), which is very close to the $BMDL_{10}$ of 112 µg/kg bw for mortality. This result indicates that the apathy observed in this study cannot be clearly separated from mortality and that the apathy might not be a sublethal effect. It shows that the study is inconclusive in this respect.

Therefore, FO considers the $BMDL_{10}$ for mortality as derived by the EFSA CONTAM Panel from this study as a more robust reference point than the dose of 25 µg TTX/kg bw that was selected by the EFSA CONTAM Panel as reference point. It is noted that the $BMDL_{10}$ for mortality might have been lower, had the observation period been longer, since prolonged observation might have revealed more mortality, also at doses at which no mortality was observed.

FO comments on the derivation of ARfD by the EFSA CONTAM Panel
The EFSA CONTAM Panel derived an ARfD of 0.25 µg/kg bw using the reference value of 25 µg/kg bw and an uncertainty factor of 100. As indicated above, FO considers the $BMDL_{10}$ for mortality as derived by the EFSA CONTAM Panel as a more robust reference point than the dose of 25 µg TTX/kg bw. Therefore, FO considers the available (limited) human data in combination with the reference point of 25 µg/kg bw as selected by the EFSA CONTAM Panel to be an insufficient scientific basis to derive an ARfD for TTX. FO

[4] In model averaging the average of the fitted models is used for calculating the BMD confidence interval, which in this case was not possible. However, the BMD confidence intervals could be calculated for the individual models, and they were all similar for this dataset, indicating that this surrogate BMDL has the usual confidence level.

would like to consider this derived ARfD by the EFSA CONTAM Panel as an indicative reference dose.

FO comments on the derivation of the CC in shellfish by the EFSA CONTAM Panel
As FO considers that there is an insufficient scientific basis for deriving an ARfD for TTX, the same holds for the derivation of a CC in shellfish, which the EFSA CONTAM Panel calculated from the derived ARfD. FO therefore would like to consider the derived CC of 44 µg/kg shellfish meat by the EFSA CONTAM Panel as indicative. FO further notes that consumption of 400 g of shellfish meat with a level of 44 µg/kg by a 70 kg person leads to an exposure which is only 450 times lower than the $BMDL_{10}$ for mortality in mice, and considers this as possibly too narrow considering the severity of the effect.

FO Assessment of the IRAS study
FO assessed the potential impact of the IRAS study on the risk assessment of TTX. The paper described a well performed *in vitro* experiment. It is noted that the results on similarity in inhibition of neuronal response *in vitro* between species could only impact on the dynamics component in the interspecies uncertainty factor. The interspecies factor of 10 is considered to be composed of a factor of 4 for differences in kinetics and a factor of 2.5 for differences in dynamics (EFSA, 2012)[5].

Front Office agrees with the conclusion of the authors that the results indicate that TTX is *in vitro* roughly equipotent in inhibiting neuronal activity in rat cells and human cells under the experimental conditions of this study. The relevance of these observations for the *in vivo* situation is not clear which hampers a direct extrapolation of the *in vitro* observations to the *in vivo* situation. For instance, one of the important topics in the present discussions on the *in vitro* to *in vivo* extrapolation of toxicity data is whether the nominal concentration in the *in vitro* system is the appropriate dose metric.

It is also not known if blocking the neuronal transmission is the only factor in expression of the effects *in vivo* after oral exposure to TTX in different species. The *in vitro* tests as used in the IRAS study have not been sufficiently verified with *in vivo* observations on a quantitative level. Therefore, FO considers the results of the IRAS study in combination with the other information on interspecies differences provided in the paper not sufficient to reduce the conventional interspecies factor for dynamics.

FO indicative approach for analysis of the available data

In the previous FO assessment (FO 2015, 2016), a preliminary reference dose of 0.13 µg/kg body weight was derived using the available human data. Assuming that the person eating the shellfish has a body weight of 60 kg and that the portion of shellfish consumed amounts to 400 grams, it was estimated that the maximum permissible concentration of TTX in shellfish meat is about 20 µg/kg (note: assuming a body weight of 70 kg would give a slightly higher maximum permissible level in shellfish meat of about 23 µg/kg). For a child with a body weight of 20 kg, this would mean that only 130

[5] FO is aware that in REACH guidance the use of allometric scaling factor for extrapolation from mouse studies of 7 as well as a remaining factor of 2.5 is recommended, resulting in a total uncertainty factor of 15 (ECHA, 2012). Also, IPCS derived ranges for allometric scaling and interspecies TK/TD factors for extrapolating from animal studies (IPCS, 2014). For the current assessment, the guidance of EFSA (2012) and IPCS (EHC240) is followed.

grams of this shellfish meat could be consumed before reaching 0.13 µg/kg bw. FO highlighted that, in view of the very limited information available, effects could not be excluded at this or even at lower concentrations in the shellfish meat.

For the current FO assessment, new information was available from an acute toxicity study in mice (Abal *et al*., 2017) and an *in vitro* neurotoxicity study in rat- and human cells (Kasteel and Westerink, 2017).

As a new indicative approach, FO considers the $BMDL_{10}$ for mortality as the most reliable reference point to estimate an indicative reference value for risk management purposes. As discussed above, FO considers the information in the *in vitro* neurotoxicity study not sufficient to adjust the uncertainty factor for interspecies differences. The $BMDL_{10}$ for mortality in mice was calculated to be 112 µg/kg bw. Besides the standard uncertainty factor of 100 (10 for interspecies differences and 10 for intraspecies differences), an extra uncertainty factor for severity of the effect (10% mortality) is needed. No default uncertainty factor is available to account for severity of effect, and its use should be considered on a case-by-case basis (EFSA, 2012). Factors up to 10 are used in the evaluation of plant protection products (EFSA, 2012). Given the severity of this effect (10% mortality) and the possibility that the calculated $BMDL_{10}$ is an overestimation of the true $BMDL_{10}$ (due to the short observation period, see discussion above) it seems appropriate to use the high end of the range, i.e. an additional factor of 10.

It may be argued that a smaller uncertainty factor for severity of effect might suffice when a dose-response curve is steep. However, a steep dose-response curve implies that at a small increase in dose, a strong increase in adverse effects (for TTX: lethality) will occur. In a case like this, deriving a reference dose using a small uncertainty factor would provide insufficient protection for effects from exposures that are only slightly above this reference dose.

The best fitting curve to the TTX mortality data appears to be somewhat steep, but that does not imply the dose-response for TTX is steep in reality. From Figure D.1 in the EFSA opinion, it can be seen that also quite shallow curves are able to describe the data, so that the true dose-response could be much shallower than the best fitting curve. In addition, the less steep curves in the same Figure D.1 show that it cannot be excluded that mortality occurs with non-negligible rates at much lower doses than the $BMDL_{10}$. Therefore, a smaller uncertainty factor for the seriousness of the effect, based on the apparent steepness of the curve, cannot be justified.

The *in vitro* dose response curve from the IRAS study cannot be extrapolated to the *in vivo* situation and thus the curve *in vivo* is not necessarily equally steep.

Based on the arguments above, FO considers that an extra uncertainty factor of 10 is needed because of the severity of the effect (10% mortality) and the possibility that the calculated $BMDL_{10}$ is an overestimation of the true $BMDL_{10}$ due to the short observation period.

Application of the uncertainty factor of 10 and the overall extrapolation factor of 100 to the $BMDL_{10}$ for mortality would give an indicative value of 0.11 µg/kg bw. Based on this indicative value, for a person weighing 70 kg eating 400 g of mussel or oyster meat, the shellfish meat may contain no more than 19.3 µg/kg TTX in order to keep the exposure to TTX less than or equal to 0.11 µg/kg body weight. A content of 19.3 µg/kg TTX means that a 20 kg child can consume no more than 115 grams of this shellfish meat.

Answers to the questions of NVWA-BuRO

1. Is there sufficient scientific basis for the health based guidance value (HBGV) that has been derived in the EFSA Opinion on TTX?
2. Is there sufficient scientific basis for the concentration TTX in shellfish meat that is not expected to lead to adverse effects in humans (critical concentration, CC) that has been identified in the EFSA Opinion on TTX?

FO considers the available (limited) human data in combination with the reference point of 25 µg/kg bw as selected by the EFSA CONTAM Panel from the Abal *et al.* (2017) study an insufficient scientific basis to derive an ARfD for TTX. The same holds for the derivation of a CC in shellfish based on this ARfD. FO would like to consider these values as indicative values.

3. Have the results from the IRAS study (Kasteel and Westerink, 2017) (sufficiently) been taken into account in the EFSA Opinion? If not, could the results give cause for a change in the HBGV or the CC?

The results from the IRAS study have not been taken into account in the EFSA Opinion, since these were not available in time for the EFSA CONTAM Panel. The IRAS study is well performed and gives insight in interspecies differences in sensitivity in inhibiting neuronal activity in rat cells and human cells *in vitro*. However, this study in combination with the other information on interspecies differences provided in the paper does not provide sufficient basis to adjust the uncertainty factor for interspecies differences used in the extrapolation from mice to humans for derivation of a HBGV.

Additional conclusions and recommendations

FO considers the $BMDL_{10}$ for mortality derived from the Abal *et al.* (2017) study by the Panel as the most reliable reference point to estimate an indicative reference value for risk management purposes. FO recommends to apply an extra uncertainty factor of 10 for severity of the effect (10% mortality) and possible overestimation of the true $BMDL_{10}$ in addition to the standard uncertainty factor of 100 (10 for interspecies differences and 10 for intraspecies differences). This approach gives an indicative reference value of 0.11 µg/kg bw. For a person weighing 70 kg eating 400 grams of mussel or oyster meat, the shellfish meat may contain no more than 19.3 µg/kg TTX in order to keep the exposure to TTX less than or equal to 0.11 µg/kg body weight. It should be noted that, based on the above, a 20 kg child should consume no more than 115 grams of this shellfish meat.

FO notes that the indicative value derived on the basis of human data (0.13 µg/kg bw; FO, 2015, 2016), the indicative reference value on the basis of the Abal *et al*. (2017) study (0.11 µg/kg bw), and the ARfD derived by the EFSA CONTAM Panel (0.25 µg/kg bw) for TTX range from 0.1 – 0.25 µg/kg bw. Thus, the differences between these derived (indicative) reference values are small, despite the different approaches in the derivation of the values. The approaches differ in the choice of the reference point and in assessment factors used.

Overall, the uncertainty in the assessments is high. As indicated before, the human data have many uncertainties, which limit their use in risk assessment. The Abal *et al.* (2017) study provides additional more information to the previous assessment, but there are considerable uncertainties in this study due to the short observation period.

FO therefore recommends to perform an APROBA analysis. With APROBA, the uncertainty in toxicity assessment can be quantitatively evaluated. With this method, it can be determined how conservative a derived health based guidance value is. In the case of TTX, APROBA can result in an uncertainty range for the dose with x% mortality in the human population (where x can be chosen at e.g. 1%, 0.1%, or 0.01%). The lower bound of that uncertainty range can be interpreted as the human dose with, at most, x% incidence. With APROBA, the proposed values for a derived health based guidance value can be better interpreted in terms of potential health risks, in this case, mortality incidence. An APROBA analysis could also indicate what type of additional information would be helpful to reduce the uncertainties in the assessment. The APROBA method is discussed in detail in IPCS (2014), and has been applied to both noncancer (Bokkers *et al.*, submitted) and cancer effects (Slob *et al.*, 2014).

Since the current assessment is based on one single acute study in female mice, extra information from an additional (acute) study in rats, especially using the lower dose ranges and longer observation periods than two hours, would be useful to validate the results from the Abal *et al.* (2017) study in female mice.

For the current FO assessment, other additional information available in the EFSA (for instance from acute studies with pharmacological TTX preparations) has not yet been considered. This could be done at a later stage.

References

Bokkers B, Mengelers M, Bakker M, Chiu W, Slob W (submitted). APROBA-Plus: A probabilistic tool to evaluate and express uncertainty in hazard characterization and exposure assessment of substances.

BuRO, 2016a. Advies over tetrodotoxines (TTX) in mosselen en oesters. Advies van de directeur bureau Risicobeoordeling & onderzoeksprogrammering van 9 maart 2016. Available via: https://www.nvwa.nl/documenten-nvwa/risicobeoordelingenvoedselveiligheid/bestand/2209661/advies-over-tetrodotoxine-ttx-in-mosselen-enoesters-09022016

BuRO, 2016b. Advies over gehaltes TTX in oesters en mosselen. Brief van 25 juni 2016 aan de inspecteur-generaal van de NVWA met het BuRO-advies over gehaltes TTX in oesters en mosselen. https://www.nvwa.nl/onderwerpen/schelpdieren/documenten/risicobeoordeling/voedselveiligheid/archief/2016m/advies-over-gehaltes-ttx-in-oesters-en-mosselen

ECHA, 2012. Guidance on information requirements and chemical safety assessment Chapter R.8: Characterisation of dose [concentration]-response for human health. ECHA-2010-G-19-EN, November 2012. Available online: https://echa.europa.eu/documents/10162/13632/information_requirements_r8_en.pdf/e153243a-03f0-44c5-8808-88af66223258

EFSA, 2009. Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission on marine biotoxins in shellfish – Saxitoxin group. EFSA Journal 2009;7(4):1019, 76 pp. doi: 10.2903/j.efsa.2009.1019

EFSA, 2012. Scientific Committee; Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels and Units in the absence of actual measured data. EFSA Journal 2012;10(3):2579. [32 pp.] doi:10.2903/j.efsa.2012.2579. Available online: www.efsa.europa.eu

EFSA, 2017a. EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), Knutsen HK, Alexander J, Barreg□ard L, Bignami M, Bruschweiler B, Ceccatelli S, Cottrill B, Dinovi M, Edler L, Grasl-Kraupp B, Hogstrand C, Hoogenboom L, Nebbia CS, Oswald IP, Rose M, Roudot A-C, Schwerdtle T, Vleminckx C, Vollmer G, Wallace H, Arnich N, Benford D, Botana L, Viviani B, Arcella D, Binaglia M, Horvath Z, Steinkellner H, van Manen M and Petersen A, 2017. Scientific opinion on the risks for public health related to the presence of tetrodotoxin (TTX) and TTX analogues in marine bivalves and gastropods. EFSA Journal 2017;15(4):4752, 65 pp. doi:10.2903/j.efsa.2017.4752

EFSA, 2017b. EFSA Scientific Committee, Hardy A, Benford D, Halldorsson T, Jeger MJ, Knutsen KH, Mo re S, Mortensen A, Naegeli H, Noteborn H, Ockleford C, Ricci A, Rychen G, Silano V, Solecki R, Turck D, Aerts M, Bodin L, Davis A, Edler L, Gundert-Remy U, Sand S, Slob W, Bottex B, Abrahantes JC, Marques DC, Kass G and Schlatter JR, 2017. Update: Guidance on the use of the benchmark dose approach in risk assessment. EFSA Journal 2017;15(1):4658, 41 pp. doi:10.29 03/j.efsa.2017.4658

IPCS, 2009. Principles and methods for the risk assessment of chemicals in food. Environmental Health Criteria 240, Chapter 5 Dose-response assessment and derivation of health-based guidance values. http://apps.who.int/iris/bitstream/10665/44065/8/WHO_EHC_240_8_eng_Chapter5.pdf?ua=1

IPCS, 2014. Guidance document on evaluating and expressing uncertainty in hazard characterization. IPCS harmonization project document ; no. 11. http://www.who.int/ipcs/methods/harmonization/uncertainty_in_hazard_characterization.pdf?ua=1

Front Office Food and Product Safety, 2016. Risk assessment of tetrodotoxins in shellfish. 07-07-2016 (English version).

Front Office Voedsel- en Productveiligheid, 2015. Beoordeling tetrodotoxines in schelpdieren 02-11-2015 (definitieve versie, herzien). Available via: https://www.nvwa.nl/documenten/risicobeoordeling/voedselveiligheid/archief/2016m/beoordeling-tetrodotoxines-in-schelpdieren.

Kasteel and Westerink, 2017. Comparison of the acute inhibitory effects of Tetrodotoxin (TTX) in rat and human neuronal networks for risk assessment purposes. Toxicology Letters 270 (2017), 12-16. http://www.sciencedirect.com/science/article/pii/S0378427417300620

OECD (Organisation for Economic Co-operation and Development), 2008. OECD Guidelines for the testing of chemicals 425. Acute Oral Toxicity-Up and Down Procedure. Organisation for Economic Co-operation and Development, Paris. pp. 1–69. Available online: https://www.oecd.org/chemicalsafety/testing/45799595.pdf

Slob W, Bakker MI, te Biesebeek JD, Bokkers BG (2014). Exploring the Uncertainties in Cancer Risk Assessment Using the Integrated Probabilistic Risk Assessment (IPRA) Approach. Risk Analysis, 34(8), 1401-1422.

Vlamis A, Katikou P, Rodrigues I, Rey V, Papazachariou AAA, Zacharaki T, Botana AM Botana LM (2015) First detection of tetrodotoxin in Greek shellfish by UPLC-MS/MS potentially linked to the presence of the dinoflagellate Prorocentrum minimum. Toxins 7, 1779-1807. Cited by EFSA, 2017.

APPENDIX I BMD analysis of apathy using surrogate method

The observations reported in the Abal et al. (2017) study are summarized in the next table.

dose	N	mortality	apathy	piloerection	paralysis	seizures	circling	squint.eyes
1000	3	3	3	0	3	3	0	0
500	5	4	5	0	2	2	2	1
250	7	4	7	1	2	2	1	0
125	9	0	9	2	0	0	0	0
75	9	0	0	0	0	0	0	0
25	9	0	0	0	0	0	0	0

The BMD analysis for apathy is summarized in the table below. Model averaging was not used here, due to the absence of partial responses. However, as the table shows, the different models results in similar BMD confidence intervals, and model averaging would not have been much different from just selecting the lowest BMDL and the highest BMDU, i.e., 73 and 126 µg/kg bw, respectively. As a technical note, the fitted models did not have a unique optimum (due to the absence of more than one partial response), and hence, the results need to be considered as approximate. In the main text these values are rounded off to 70 and 125 µg/kg bw.

BMD analysis for apathy

model	No.par	log-likelihood	accepted	AIC	BMDL	BMDU
null	1	-28.68		59.36	NA	NA
full	6	0		12	NA	NA
two.stage	3	-7.58	no	21.16	NA	NA
log.logist	3	0	yes	6	73.2	120
Weibull	3	0	yes	6	79.4	126
log.prob	3	0	yes	6	72.6	122
gamma	3	-0.1	no	6.2	NA	NA
logistic	2	0	yes	4	83.5	122
probit	2	0	yes	4	92	126
LVM_Exp: m3-	3	0	yes	6	71.7	113
LVM_Hill: m3-	3	-0.02	no	6.04	NA	NA

PROAST version: 64.1

no covariate

BMR: 0.1 extra risk

constraint: no