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Assessment of the genotoxicity of the cyanotoxin cylindrospermopsin

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Beoordeling van de genotoxiciteit van het blauwalgtoxine cylindrospermopsine

Samenvatting

In periodes van warm weer kan in oppervlaktewater bloei optreden van cyanobacteriën. Deze bacteriën kunnen toxines produceren (cyanotoxines of blauwalgtoxines) die in het water terecht kunnen komen. Het gebruik van dit verontreinigde water voor het besproeien van landbouwgewassen of het drenken van vee kan schadelijk zijn voor mensen en dieren. Ook consumptie van verontreinigd voedsel (bijvoorbeeld schaal- en schelpdieren) of drinkwater kan schadelijk zijn voor mensen. Om het risico op schadelijke effecten beter te kunnen inschatten is het belangrijk om te weten of deze blauwalgtoxines genotoxisch zijn, oftewel of ze DNA kunnen beschadigen. Bureau Risicobeoordeling & onderzoek (BuRO) heeft het Front Office Voedsel- en Productveiligheid (FO) gevraagd om te beoordelen of het blauwalgtoxine cylindrospermopsine (CYN) genotoxisch is. Het FO heeft hiertoe een literatuuronderzoek uitgevoerd.

Uit dit onderzoek blijkt dat CYN beschouwd moet worden als een genotoxische stof. Op basis van de resultaten van *in vitro* micronucleus testen en micronucleus testen in ratten kan worden geconcludeerd dat CYN een clastogene stof is. Dit betekent dat CYN structurele chromosoomafwijkingen kan veroorzaken. CYN moet in het lichaam waarschijnlijk omgezet worden in actieve metabolieten voordat het clastogene effecten heeft. De beschikbare *in vitro* studies naar genmutaties (mutageniteit) leveren geen bewijs voor mutagene eigenschappen van CYN. Echter, de concentraties die in deze studies werden gebruikt waren mogelijk te laag. In combinatie met de positieve resultaten van de *in vivo* comet assays, die zowel clastogene als mutagene eigenschappen van een stof kunnen detecteren, kan mutageniteit van CYN niet worden uitgesloten.

Subject
BuRO is preparing a risk assessment of blue-green algae toxins in surface water for irrigation of food crops and cattle drenching. A well-founded hazard characterization of the blue-green algae toxins is necessary for this. For this aim, an assessment of the genotoxicity of cylindrospermopsin has been requested.

Question
Should cylindrospermopsin – further referred to as CYN - be considered as a genotoxic substance?

Conclusions
CYN should be considered as a genotoxic substance. Based on the results of *in vitro* assays and *in vivo* micronucleus assays in rats, it can be concluded that CYN is a clastogenic substance (i.e. CYN can cause structural chromosome aberrations), most likely after metabolic activation. The *in vitro* gene mutation studies identified do not provide evidence for mutagenic properties of CYN. However, the concentrations used in these assays might have been too low. In combination with the positive results of the *in vivo* comet assays, which can detect both clastogenic and mutagenic properties of a test article, mutagenicity of CYN cannot be ruled out.

Introduction
During warm periods, blooms of cyanobacteria (blue-green algae) can be observed in surface waters. Cyanobacteria can produce toxic substances (cyanotoxins) which can end up in the water. Use of contaminated water for spraying crops or drenching of farm animals could be harmful to humans and livestock. Also consumption of contaminated food (e.g. shellfish and crustaceans) or drinking water could be harmful to humans.

Cylindrospermopsin (CYN) inhibits protein synthesis and is toxic to a variety of tissues/organs (liver, kidneys, spleen and lungs), it may affect spermiogenesis and is suspected of genotoxicity.
In January 2019, the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) published a risk assessment report on CYN (ANSES, 2019). ANSES derived for this toxin a subchronic toxicological reference value (TRV) for the oral route[1]. The TRV for CYN amounted to 0.14 µg/kg bw/day, and was derived from a Lowest Observed Adverse Effect Level (LOAEL) for adverse liver effects observed in a 90-day oral toxicity study in mice, published by Chernoff et al. (2018).
In addition, in November 2019, the World Health Organization (WHO) published a draft background document on CYN for the development of Guidelines for drinking water quality and Guidelines for safe recreational water environments (WHO, 2019). At the moment, the WHO report is a draft version open for public review.

In May 2020, by request of BuRO, RIVM performed Bench Mark Dose (BMD) analyses for CYN (RIVM, 2020). Starting point for the assessment was the assumption that the study by Chernoff et al. (2018) is the critical study for the derivation of a Point of Departure (PoD) for a (sub)chronic Health Based Guidance Value (HBGV) for CYN. Based on this subchronic study, a Bench Mark Dose Lower Confidence Limit (BMDL) of 9.4 µg/kg bw (related to an 8.7%[2] increase in 'relative liver weight') was derived (RIVM, 2020). Also, an Acute Reference Dose (ARfD) of 0.5 µg/kg bw for CYN was derived, based on a No Observed Adverse Effect Level (NOAEL) of 50 µg/kg bw/day obtained from a subacute

[1] In a previous evaluation (RIVM, 2020), this toxicological reference value was erroneously referred to as 'tolerable daily intake'.
[2] The BMR was derived based on the BMR 1 SD approach, but performed on the log-scale. For substantiation see Slob et al. (2017).

toxicity study in mice (RIVM and WFSR, 2020). The critical effect was lipid infiltration in liver cells. To this NOAEL an uncertainty factor of 100 was applied, which resulted in an ARfD of 0.5 µg/kg bw. ANSES (2019) and WHO (2019) did not address the possibility and need for an ARfD for CYN.

The current evaluation assesses the genotoxicity of CYN. ANSES (2019) concluded that whereas *in vitro* studies supported the existence of a genotoxic effect of CYN, the *in vivo* results were very fragmented and that it was not possible to conclude on the genotoxic potential of CYN. WHO (2019) concluded that some *in vitro* and *in vivo* studies suggest that CYN may have genotoxic potential, but indicated that the database is limited. After the publication of the (draft) opinions by ANSES and WHO, new studies of genotoxicity with CYN were published. These studies, as well as the studies considered by ANSES and WHO are included in the current evaluation.

Methodology

A literature search was performed recently (RIVM and WFSR, 2020) to identify scientific information on the toxicity (including genotoxicity) of CYN. The search was conducted in Embase, Pubmed and Scopus using "cylindrospermopsin" as search term (in title, abstract, keywords). This gave 523 hits in Pubmed, 819 hits in Scopus and 625 hits in Embase. The references retrieved from searching the three databases were extracted and combined into an Endnote file and duplicates were removed. In total 845 unique references were found. Based on screening of the titles, 26 references on genotoxicity were identified that were considered for inclusion in this evaluation. Comparison with the references on genotoxicity listed by ANSES (2019) and WHO (2019) did not identify additional references from these documents.

Toxicological data

Below, the available studies on genotoxicity are described. A summary table of all *in vitro* and *in vivo* assays is presented in Annex 1 and 2, respectively. These are all studies from public literature. Some authors reported that their studies were performed in accordance with a relevant Organisation for Economic Cooperation and Development (OECD) test guideline. If this is the case, it is mentioned below and/or in the Annexes. For other studies, it is checked whether the study protocol is comparable to the relevant test guideline.

<u>Studies on genotoxicity *in vitro*</u>

Bacterial reverse mutation assay

In a bacterial reverse mutation assay with CYN (purity 95%; up to 10 µg/mL) in *Salmonella typhimurium* strains TA97A, TA98, TA100, TA102 and TA104, negative results were obtained with and without rat liver S9 (Puerto et al., 2018). Also Sieroslawska (2013) obtained negative results with CYN (pure form, exact purity unknown; up to 10 µg/mL) in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2 *uvr*A and WP2 [pKM101] with and without rat liver S9 in a microplate format bacterial reverse mutation assay. No signs of precipitation or of toxicity to the bacterial strain were observed in these assays. According to Puerto et al. (2018), their assay was performed according to the principles of OECD test guideline No. 471 (OECD, 1997) with modifications (not further specified). It is noted that strain TA104 was used instead of TA1535. The concentrations used in this study cannot be compared to recommended concentrations in the guideline, since information is lacking to express them in µg/plate or µL/plate. For the microplate format assay, no OECD test guideline is available (yet).

The concentrations used in these assays might have been too low since they did not induce (sufficient) cytotoxicity.
Two extracts from bacterial blooms containing CYN gave positive results in the microplate format bacterial reverse mutation assay by Sieroslawska (2013). However, since the concentrations tested were very low (equal to up to 0.89 ng CYN/mL) it is likely that other components from the extracts are responsible for the positive results obtained (Sieroslawska, 2013).

Mammalian gene mutation assay
CYN (purity 95%) was negative in a mouse lymphoma thymidine-kinase assay at concentrations up to 0.675 µg/mL in the absence and presence of rat liver S9 (Puerto et al., 2018). The study was reported to be performed in accordance with OECD test guideline No. 490 (OECD, 2016a). The concentrations used were based on a pilot experiment (data not shown). The relative total growth (RTG) of the cells at the highest concentration tested in the two experiments was 75 and 82% (-S9, 4 h exposure), 37 and 29% (+S9, 4 h exposure) and 30 and 26% (-S9, 24 h exposure). According to the guideline a RTG between 10 – 20% should be aimed for in case the highest dose tested is based on cytotoxicity, so the concentration used in this experiment might have been too low.

Micronucleus assay
Six *in vitro* micronucleus assays have been conducted with CYN (Bazin et al. 2010; Lankoff et al., 2007; Sieroslawska and Rymuszka, 2015; Puerto et al., 2018; Hercog et al., 2017; Štraser et al., 2011a). The design of the studies was generally comparable to OECD test guideline No. 487 (OECD, 2016b), except for the study by Sieroslawska and Rymuszka (2015) that used carp cells instead of mammalian cells. Positive results were obtained in human intestinal cells (Caco-2), human liver cells (HepG2 and HepaRG), mouse lymphoma cells (L5178YTk$^{+/-}$) [in the presence of S9 only] and carp leukocytes. Negative results were obtained in Chinese hamster ovary (CHO) K1 cells (Lankoff et al., 2017).

In the study of Bazin et al. (2010), in Caco-2 cells, statistically significant and concentration-dependent increases in the frequency of binucleated cells with micronuclei (MNBNC) were observed after exposure to CYN. The increases were greater in undifferentiated compared to differentiated cells. The frequency of mononucleated cells with micronuclei (MNMC) was also increased but this was not concentration-dependent. No alterations in the frequency of polynucleated, mitotic or apoptotic cells were observed. In HepaRG cells, the MNBNC frequency was statistically significantly increased in differentiated cells exposed to CYN compared to the corresponding control, except at the highest concentration. This is most likely due to cytotoxicity at the highest concentration. No statistically significant changes in the frequency of MNMC and of polynucleated, apoptotic or mitotic cells was observed. After preincubation for 2 h with ketoconazole, an inhibitor of cytochrome P450 3A4 as well as other P450 isoenzymes, the number of cells with micronuclei was reduced in Caco-2 cells, whereas no induction of cells with micronuclei was observed in HepaRG cells. This suggests that metabolic activation by CYP is required for the genotoxic response (Bazin et al., 2010).

In L5178YTk$^{+/-}$ cells, CYN induced a statistically significant increase in the frequency of binucleated cells with micronuclei (BNMN) but only in the presence of S9 (Puerto et al., 2018).
In HepG2 cells, CYN statistically significantly induced the frequency of micronucleated cells at the highest concentration tested (0.5 µg/mL) (Hercog et al., 2017). Also in another micronucleus assay in HepG2 cells, CYN statistically significant increased the

frequencies of micronucleated cells (Štraser et al., 2011). The frequency of cells with nuclear buds was statistically significantly increased at 0.05 and 0.5 µg/mL. An increase in the frequency of cells with nuclear bridges was detected at 0.05 and 0.5 but reached statistical significance only at 0.05 µg/mL CYN (Štraser et al., 2011).

In a micronucleus test in CHO K1 cells, no increase in cells with micronuclei was observed after exposure to up to 2 µg/mL in the presence and absence of rat liver S9. The mitotic index values decreased in a concentration- and time-dependent manner (Lankoff et al., 2007).
In addition, positive results were obtained for CYN in a micronucleus test in a carp cell line (Sieroslawska and Rymuszka, 2015), but it is noted that this cell line is less relevant for human risk assessment.

Comet assay
Eight studies using single cell gel electrophoresis (comet) assays were performed with CYN (Fessard & Bernard 2003, Fonseca et al., 2013, Puerto et al., 2018, Sieroslawska and Rymuszka, 2013, Štraser et al., 2011, Štraser et al., 2013, Hercog et al., 2017, Humpage et al., 2005). No OECD test guideline is currently available for the *in vitro* comet assay.

CYN statistically significantly induced DNA damage in HepG2 cells exposed to concentrations up to 0.3-0.5 µg/mL (Fonseca et al., 2013; Štraser et al., 2011, 2013a; Hercog et al., 2017) as well as in mouse hepatocytes exposed to up to 0.5 µg/mL (Humpage et al., 2005). In the latter experiment, no induction of DNA damage was observed when the cells were pretreated with 50 µM SKF525A (a broad spectrum monooxygenase inhibitor) or 100 µM omeprazole (an inhibitor of mainly P450 3A4 and 2C19). Štraser et al., 2013a performed an enzyme modified comet assay using the enzyme formamidopyrimidine DNA glycosylase (Fpg) that excises oxidized DNA bases. After normalizing the mean values of % tail DNA to the corresponding vehicle controls, there was no significant difference between undigested and Fpg-digested samples, showing that the DNA damage caused by CYN was unlikely to be due to oxidative stress. Also, positive results were obtained for CYN in a comet assay in a carp cell line (Sieroslawska and Rymuszka, 2013), but it is noted that this cell line is less relevant for human risk assessment.

CYN did not statistically significantly induce DNA damage in CHO-K1 cells exposed to 0.5 and 1 µg/mL for 24 h (Fessard and Bernard, 2003). Cell viability was not affected, whereas growth inhibition (>50%) was observed at both test concentrations.
In the study of Puerto et al. (2018), negative results were obtained in a standard comet assay with Caco-2 cells exposed to 0.625 - 2.5 µg/mL CYN for 24 or 48 h. Negative results were also obtained by Puerto et al. (2018) in an enzyme modified comet assay using the enzymes Endonuclease III and formamidopyrimidine DNA glycosylase, which detect oxidized bases. The test concentrations were based on a previous cytotoxicity test, but no actual cytotoxicity data for this study were provided.

Double strand breaks (γH2AX assay)
Induction of DNA double strand breaks by CYN was reported in four γH2AX studies (Štraser et al., 2013b; Hercog et al., 2020a; Hercog et al., 2020b; Huguet et al., 2019). In this assay, the induction of DNA double strand breaks is measured indirectly by measuring the induction of γH2AX formation. An OECD test guideline for double strand breaks (γH2AX assay) is currently not available.

In the study of Štraser et al. (2013b) CYN significantly induced the γH2AX signal in HepG2 cells exposed to 0.5 µg/mL CYN for 72 h but not for 24 h. At 0.125 µg/mL CYN significantly decreased the γH2AX signal compared to the control, the same was observed after 24 h of exposure to 0.125 and 0.25 µg/mL CYN. This could be due to DSB repair processes or the protein synthesis inhibition by CYN. Also in another study in HepG2 cells, positive results were obtained after 72 h of exposure to CYN (Hercog et al., 2020b). In a study by the same authors using a HepG2 3D (spheroid) model, negative results were obtained for CYN (Hercog et al., 2020a).
In the study of Huguet et al. (2019), a concentration dependent increase (statistically significant from 12.5 µM (5.2 µg/mL) onwards) in γH2AX positive cells was observed in undifferentiated HepaRG cells.

Other studies
Since the literature search was aimed at the identification of genotoxicity studies with CYN, studies on cell cycle analysis and gene expression were not included. However, some information was found in the papers describing genotoxicity studies. A summary of this information is provided below. This does not give a full overview of the information available on these aspects.

In HepG2 cells exposed to 0.5 µg/mL CYN, the expression of *CYP1A1* and *CYP1A2* genes was upregulated. Exposure to CYN did not induce any changes in the mRNA expression of *P53*. This is not unexpected, since in response to DNA damage, p53 protein is predominantly activated through its phosphorylation by DNA damage responsive kinases and, to a lesser extent, through the up-regulation of gene expression. The expression of its target genes *CDKN1A*, *GADD45A*, and *MDM2* was significantly induced (Štraser et al., 2011). *CDKN1A* and *GADD45A* are induced upon DNA damage and mediate cell cycle arrest, while *MDM2* is involved in the regulation of stress-induced p53 activity.

Štraser et al. (2013b) showed that CYN reduced cell viability of HepG2 cells in a concentration and time dependent manner as demonstrated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay. Because no increased lactate dehydrogenase (LDH) leakage occurred, the authors concluded that the reduced cell number is due to decreased cell proliferation rather than cytotoxicity. This was confirmed by a concentration and time dependent decrease in the percentage of cells in which the cell proliferation marker Ki67 was detected. This protein is absent in resting (G0) cells. Štraser et al. (2013b) indicated that the decrease in Ki67 could also partly be due to inhibition of protein synthesis by CYN. Analysis of the cell-cycle using flow-cytometry showed induction of cell cycle arrest in the G0/G1 phase after 24 h of exposure and in S phase after prolonged exposure (72 and 96 h).

In the study of Huguet et al. (2019), a concentration-dependent decrease in cell counts was observed in HepaRG cells after 24 h of CYN treatment. Cell cycle arrest was demonstrated by accumulation of cells in the G2/M phase and an increase in phospho-H3-positive cells (markers of aneuploidy and mitotic block). There was also an accumulation of cells in the S phase, combined with a decrease in the expression of *PCNA* (a gene involved in DNA replication) and *CDKN2C*, a gene controlling the G1/S cell transition.

Hercog et al. (2017) showed that exposure of HepG2 cells to 0.5 µg/mL CYN for 24 h deregulated the expression of genes involved in xenobiotic metabolism (upregulation of *CYP1A1* and *CYP1A2*, downregulation of *NAT2*), immediate early response/signaling (upregulation of *FOS*, *JUN and TGFB2*) and DNA damage response (upregulation of *MDM2*, *CDKN1A*, *GADD45A* and *ERCC4*).

Hercog et al (2020a) showed that in HepG2 spheroids exposed to CYN (⩾0.25 µg/mL) for 72 h, the number of cells in the G0/G1 phase was increased, together with the reduction of the number of cells in the S phase. In these HepG2 spheroids, no S phase arrest was seen in contrast to the studies by Huguet et al. (2019) and Štraser et al. (2013b). CYN upregulated the expression of *CYP1A1* and *CYP3A4* suggesting their role in the activation of the toxin, while the transcription of *CYP1A2* was not significantly affected. Another phase I enzyme, *ALDH3A1*, was also strongly upregulated. CYN upregulated the mRNA level of all studied phase II enzymes (*NAT1, SULT1B1, SULT1C2, UGT1A1, UGT2B7*), with the exception of *NAT2*, which was downregulated. The DNA damage response genes (*GADD45a, CDKN1A* and *ERCC4*) were all upregulated by CYN. Downregulation of cell proliferation genes (*PCNA* and *TOP2α*) by CYN indicated the toxin's negative impact on the cell proliferation.

Hercog et al. (2020b) showed that exposure of HepG2 cells to 0.5 µg/mL CYN for 24 h did not affect cell viability, whereas cell viability was reduced after 72 h of exposure. Gene expression of a selection of genes was analyzed after 24 h of exposure by quantitative real-time PCR. *CYP1A1* (involved in metabolism of CYN) was upregulated. P53 was slightly downregulated. *CDKN1A and GADD45A* (involved in DNA damage) were upregulated, whereas *MDM2* (also involved in DNA damage) was unresponsive to the exposure to CYN. From the genes involved in oxidative stress response that were analyzed, *GCLC* and *GPX1* was upregulated, *SOD1A* was downregulated and *CAT* and *GSR* were unaffected.

Studies of genotoxicity *in vivo*

Micronucleus and comet assays

Two publications describe combined micronucleus/comet assays with CYN (Bazin et al., 2012; Diez-Quijada et al., 2019). In addition, one publication describes a combined micronucleus/comet assay with a combination of CYN and microcystin-LR (MC-LR) (Diez-Quijada et al., 2020). These studies are discussed below.

Bazin et al. (2012) administered to male Swiss Albino mice (n=3 per group) a single dose of 0, 50, 100, 200 µg/kg bw CYN (purity 98%) i.p. or a single dose of 0, 1, 2, 4 mg/kg bw CYN via gavage for a combined micronucleus and comet assay. Animals were killed 24 h after administration. Clinical signs were monitored during the treatment period. Samples of bone marrow, liver, kidneys, ileum and colon were collected.
After i.p. administration, no mortality was observed; one mouse from the highest dose group had a pale liver. Histological analysis revealed cell death in liver and kidneys in the 100 and 200 µg/kg bw dose groups. For the micronucleus assay in bone marrow cells, 2,000 polychromatic erythrocytes (PCE) and at least 1,000 normochromatic erythrocytes (NCE) per animal were analyzed for micronuclei. Cytotoxicity was estimated by the ratio PCE and NCE. The analyzes were performed in accordance with OECD test guideline No. 474 (OECD, 2016c). No statistically significant increases in cells with micronuclei were observed in the bone marrow samples. No direct evidence of bone marrow exposure was provided since the results of the cytotoxicity analyses were not provided in the publication.
The comet assay was performed in blood, bone marrow, kidney, liver, ileum and colon cells. A statistically significant increase in %tail DNA was observed in the colon at 100 and 200 µg/kg bw ($P<0.05$). This was however not dose-related as the highest dose resulted in a lower response in colon cells than the mid-dose (12.2 versus 24.7% tail DNA). No cytotoxicity was observed in the colon.

After oral administration, one mouse treated with 4 mg/kg bw was moribund after 24 h and examination revealed a dark red liver and intestinal hemorrhage. Another mouse had an intestinal bleeding and liquid stool. One of the mice treated with 2 mg/kg bw died. Histological analysis showed apoptosis in both liver and kidney tissues at 2 and 4 mg/kg. In addition, in some mice, apoptosis of lymphocytes within Peyer's patches in duodenum and jejunum was observed at these doses. For the micronucleus assay in colon crypts, at least 1,000 cells per mouse were scored. No statistically significant increases in cells with micronuclei were observed.
The comet assay was performed in blood, bone marrow, kidney, liver, ileum and colon cells. A statistically significant increase in % tail DNA in bone marrow cells was observed at 1 and 2 mg/kg bw. At 4 mg/kg, no significant increase in %tail DNA was observed, but there was a high variability between mice. Again, no dose-related increases were observed in bone marrow. In colon cells, a statistically significant increase in % tail DNA compared to the negative control was observed at 4 mg/kg bw. No cytotoxicity was observed in bone marrow cells or colon cells.
When the study design of the comet assay was compared to OECD test guideline No. 489 (OECD, 2016d), a few deviations were noted. Differences included three animals per dose group instead of five, a single dose instead of two or more treatments over a duration of two or more days, sacrificing after 24 h instead of after 2-6 h or at T_{max} and the scoring of 50 cells instead of 150 cells.

In the study of Diez-Quijada et al. (2019), male Wistar rats (3-5 per group) were exposed to 0, 7.5, 23.7 or 75 µg/kg bw CYN (purity 98%) by gavage at 0, 24 and 45 h. Animals were killed 3 h after the last administration. Clinical signs and bodyweight were recorded during the exposure period, and blood and liver and stomach samples were collected for histopathology. Bone marrow samples of both femurs of each animal were collected for the micronucleus assay and, per animal, 500 erythrocytes were counted for PCE:total erythrocytes and PCE:NCE ratios and 5,000 PCE for micronuclei. The micronucleus assay was performed in accordance with OECD test guideline 474 (OECD, 2016c). The comet assay was performed in blood, liver and stomach cells and in accordance with OECD test guideline 489 (OECD, 2016d).
No mortalities or signs of toxicity were observed. However, significantly altered gastric mucus secretion and mucosal damage was observed in the treated rats. In addition, modifications in the nuclei structure of liver cells and proliferation of smooth endoplasmic reticulum were observed at all doses. At the highest dose a high mitotic frequency was observed. At the low and mid dose, the degenerative changes were found at perilobular hepatocytes while at the high dose changes in both perilobular and centrilobular hepatocytes were observed.
In the micronucleus assay, statistically significant increases in percentage of cells with micronuclei (%MN) among the immature erythrocytes were observed for all dose groups, but no dose-dependency. The percentage of cells with micronuclei was highest at 7.5 µg/kg bw (2.19%; $p<0.001$) and decreased with higher doses, 1.72% at 23.7 µg/kg bw and 1.65% at 75 µg/kg (both $p<0.01$). Dose-dependent decreases in PCE:total erythrocytes and PCE:NCE ratios compared to the negative control were observed, that became statistically significant at 75 µg/kg bw ($p<0.01$), indicating cytotoxicity at the highest dose.
The comet assay was negative in all tissues at all doses. Histopathological changes in the liver and stomach of the animals evidenced exposure of these tissues.

In a follow up study by the same authors with a comparable design, male and female Wister rats were administered 0 + 0, 7.5 + 75, 23.7 + 237 or 75 + 750 µg/kg bw CYN + MC-LR by gavage at 0, 24 and 45 h (Diez-Quijada et al., 2020). Similar results as with CYN only (Diez-Quijada et al., 2019) were obtained. There were significant increases

compared to negative control in %MN in immature erythrocytes in all treated rats ($p<0.01$) without dose-dependency. At the same time, significant decreases ($p<0.01$) were observed in PCE:total erythrocytes and PCE:NCE ratios observed in both sexes at the highest dose compared to controls, indicating cytotoxicity. The comet assay was negative in all tissues at all doses. Histopathological changes in the liver and stomach of the animals evidenced exposure of these tissues.

In addition, a comet assay has been performed by Dordevic et al. (2017). Male Wistar rats were given a single i.p. dose of 0 or 79.80 µg/kg bw CYN (purity 98%) or of 0, 1500, 3000, 6000 and 12,000 µg/kg bw methanolic extract of *C. raciborskii* (containing 6.65 µg CYN/mg extract) corresponding to 0, 9.97, 19.95, 39.90 and 79.80 µg/kg bw CYN. Animals were killed 24 or 72 h after treatment and liver cells were analyzed for tail length, %DNA in tail and tail moment.
Both after administration of CYN and the extract, statistically significant increases in all three parameters were observed compared to negative control at both timepoints. The increases were generally dose-dependent.
When the study design was compared to the OECD test guideline 489, a few differences were observed. These include a single dose instead of two or more treatments over a duration of two or more days, sacrificing after 24 or 72 h instead of after 2-6 h or at T_{max} and the scoring of 100 comet images.

Other assays
Two additional *in vivo* assays were identified, a ^{32}P-postlabelling assay in male white Quackenbush mice (Shaw et al., 2000) and a DNA strand breaks assay in Balb/c mice (Shen et al., 2002). Both assays showed positive results. The study of Shaw et al. lacked a detailed description and the test material, doses used and tissues collected were unclear and therefore this study cannot be used to assess the genotoxicity of CYN. The design of the DNA strand breaks assay (Shen et al., 2002) is not in accordance with accepted test guidelines for assessment of genotoxicity; nevertheless, the results can be used as supportive data.

Discussion
The available *in vitro* studies do not provide evidence for mutagenicity of CYN, since CYN gave negative results in bacterial reverse mutation assays (Puerto et al., 2018; Sieroslawska, 2013) and in a mouse lymphoma assay (Puerto et al., 2018). However, the concentrations used in these assays might have been too low since they did not induce (sufficient) cytotoxicity. Two extracts from bacterial blooms containing CYN gave positive results in a bacterial reverse mutation assay. However, since the concentrations tested were very low (equal to up to 0.89 ng CYN/mL) it is likely that other components from the extracts are responsible for the positive results (Sieroslawska, 2013).

In *in vitro* micronucleus assays positive results were obtained in human intestinal cells (Caco-2), human liver cells (HepG2 and HepaRG), mouse lymphoma cells (L5178YTk+/-) [in the presence of S9 only] and carp leukocytes (although it is noted that this cell line is less relevant for human risk assessment). Preincubation with the P450 inhibitor ketoconazole resulted in a reduction of the number of cells with micronuclei in Caco-2 cells, and in negative results in HepaRG cells (Bazin et al., 2010). Negative results were obtained in Chinese hamster ovary (CHO) K1 cells in the absence and presence of metabolic activation (Lankoff et al., 2017). These results indicate that CYN has the potential to cause clastogenic effects, most likely after metabolic activation as shown by the experiment with the P450 inhibitor.

The results from the *in vitro* comet assays were less consistent. CYN statistically significantly induced DNA damage in a comet assay in HepG2 cells (Fonseca et al., 2013; Štraser et al., 2011, 2013a; Hercog et al., 2017), mouse hepatocytes (Humpage et al., 2005) and – less relevant - carp leukocytes (Sieroslawska and Rymuszka, 2013). In mouse hepatocytes, no induction of DNA damage was observed when the cells were pretreated with 50 µM SKF525A (a broad spectrum monooxygenase inhibitor) or 100 µM omeprazole (an inhibitor of mainly P450 3A4 and 2C19)(Humpage et al., 2005). The enzyme-modified comet assay performed by Štraser et al. (2013a) did not provide an indication for a role of reactive oxygen species in the DNA damage caused by CYN. In CHO K1 cells negative results were obtained, as was the case with the micronucleus assay. In addition, CYN was negative in both the standard comet assay as well as an enzyme modified comet assay in Caco-2 cells (Puerto et al., 2018). It should be noted that the *in vitro* comet assay is not a generally accepted genotoxicity assay.

Several γH2AX assays showed an induction of DNA double strand breaks and are supportive for the clastogenicity of CYN (Štraser et al., 2013b; Hercog et al., 2020b; Huguet et al., 2019. Negative results were obtained in a HepG2 model (HepG2 spheroids; Hercog et al., 2020a)). This difference can possibly be explained by lower intracellular concentrations of the test compound.

Based on the limited information obtained from cell cycle and gene expression analyses that was considered for the current evaluation, it seems likely that CYN can cause cell cycle arrest and can deregulate genes involved in metabolism, cell proliferation and DNA repair. More information on these aspects is available in public literature but was not considered for the current evaluation.

CYN showed positive as well as negative results in both the micronucleus assay and comet assay *in vivo*.
In mice, CYN was positive in the comet assay but negative in the micronucleus assay, both after oral and after i.p. administration (Bazin et al., 2012). The authors indicated that the primary DNA damage detected in the comet assay might have been repaired or not yet fixed into stable lesions that could be detected in the micronucleus assay as an explanation for the difference in outcome between the two assays. This micronucleus assay in mice was performed with tissues collected at 24 h after a single dose, whereas the micronucleus assay in rats performed by Diez-Qujiada et al. (2019) was conducted with tissues sampled 48 h after the first of three doses and gave positive results. The positive results were confirmed in the micronucleus assay with a combination of CYN and MC-LR (Diez-Quijada et al., 2020). On the other hand, the comet assays performed by Diez-Quijada et al. (2019 and 2020) in rats after oral administration showed negative results. It is noted that the oral dose used by Bazin et al. (2012) was much higher (1-4 mg/kg bw compared to 7.5-75 µg/kg bw) and was in the lethal dose range. Dordevic et al. (2017) used the i.p. route and also obtained positive results in the comet assay in rats.

Based on the results of the *in vitro* assays and the *in vivo* micronucleus assays in rats, it can be concluded that CYN is a clastogenic compound. Based on the results of the *in vivo* comet assays, which can detect both clastogenicity and mutagenicity, after i.p. administration in rats and mice, mutagenicity cannot be ruled out.

Conclusion
CYN should be considered as a genotoxic substance. Based on the results of *in vitro* assays and *in vivo* micronucleus assays in rats, it can be concluded that CYN is a clastogenic substance (i.e. CYN can cause structural chromosome aberrations), most

likely after metabolic activation. The *in vitro* gene mutation studies identified do not provide evidence for mutagenic properties of CYN. However, the concentrations used in these assays might have been too low. In combination with the positive results of the *in vivo* comet assays, which can detect both clastogenic and mutagenic properties of a test article, mutagenicity of CYN cannot be ruled out.

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Annex 1. In vitro genotoxicity tests with cylindrospermopsin (CYN)

Test article	**Assay**	**Species**	**Time of exposure**	**Concentrations**	**Results**	**Reference**	**Remarks study protocol**
Assays with CYN							
CYN (95% purity)	Bacterial reverse mutation (Ames) assay	*S. typhimurium* TA97a, TA98, TA100, TA102 and TA104	Not reported	0.625–10 µg/mL, ±S9	Negative[a]	Puerto et al., 2018	In accordance with OECD guideline 471 (1971) with (unspecified) modifications.
CYN (purity unknown)	Bacterial reverse mutation (Ames) assay	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2 uvrA and WP2 [pKM101]	90 min preincubation + 48 h	0.312, 0.625, 1.25, 2.5, 5 and 10 µg/mL ±S9	Negative[b]	Sieroslawska, 2013	Ames microplate format mutagenicity assay.
CYN (purity 95%)	Mammalian gene mutation (mouse lymphoma assay)	L5178Y $Tk^{+/-}$ cells	4 h (± S9 24 h (-S9)	0.042, 0.084, 0.168, 0.338, 0.675 µg/mL	Negative[c]	Puerto et al., 2018	In accordance with OECD test guideline No. 490
CYN (purity >98%)	Micronucleus assay	Caco-2 cells (undifferentiated) Caco-2 cells (differentiated) HepaRG cells (undifferentiated) Caco-2 cells (differentiated)	24 h	0, 0.5, 1, 1.25, 1.5, 1.75 and 2 µg/mL, ± ketoconazole.	Positive[d]	Bazin et al. 2010	In accordance with OECD test guideline No. 487

Test article	Assay	Species	Time of exposure	Concentrations	Results	Reference	Remarks study protocol
CYN (purified from cultures of *C. raciborskii*; purity not reported)	Micronucleus assay	CHO K1 cells	3 h, 16 h or 21 h	0, 0.05, 0.1; 0.2; 0.5, 1, 2 µg/mL, ±S9	Negative[e]	Lankoff et al., 2007	Comparable to OECD test guideline No. 487
CYN (Purity unknown)	Micronucleus assay	Common carp (*Cyprinus carpio L.*) leucocyte cell line (CLC)	24 h	0, 0.1, 0.5 or 1 µg/mL	Positive[f]	Sieroslawska and Rymuszka, 2015	Assay performed in fish cell line instead of mammalian cell line.
CYN (purity 95%)	Micronucleus assay	L5178Y $Tk^{+/-}$ cells	24 h (-S9) 4 h (+S9)	0–1.35 µg/mL, -S9; 0–2 µg/mL, +S9	Positive[g]	Puerto et al., 2018	In accordance with OECD test guideline No. 487
CYN (purity unknown)	Micronucleus assay	HepG2 cells	24 h	0, 0.01, 0.05, 0.1 and 0.5 µg/mL, ±1 µg/mL MCLR	Positive[h]	Hercog et al., 2017	Comparable to OECD guideline No. 487.
CYN (purity unknown)	Micronucleus assay	HepG2 cells	24 h	0, 0.005, 0.05, and 0.5 µg/ml	Positive[i]	Štraser et al., 2011	Comparable to OECD guideline No. 487.
CYN (purified from cultures of *C. raciborskii* , purity unknown)	Comet assay	Chinese hamster ovary cells (CHO K1)	24 h	0.5 and 1 µg/mL	Negative[j]	Fessard & Bernard 2003	No OECD test guideline available

Test article	Assay	Species	Time of exposure	Concentrations	Results	Reference	Remarks study protocol
CYN (puritified from cultures of *C. raciborskii*, purity unknown)	Comet assay	HepG2 cells	24 h 48 h 72 h	0, 0.03, 0.15, and 0.3 µg/mL	Positive[k]	Fonseca et al., 2013	No OECD test guideline available
CYN (purity 95%)	Comet assay	Caco-2 cells	24 h 48 h	0, 0.625, 1.25, and 2.5 µg/mL	Negative[l]	Puerto et al., 2018	No OECD test guideline available
CYN (purity unknown)	Comet assay	Common carp (*Cyprinus carpio* L.) leucocyte cells (CLC)	18 h	0 and 0.5 µg/mL	Positive[m]	Sieroslawska and Rymuszka, 2013	No OECD test guideline available
CYN (purity unknown)	Comet assay	HepG2 cells	4 h 12 h 24 h	0, 0.005, 0.01, 0.05, 0.1, and 0.5 µg/mL	Positive[n]	Štraser et al., 2011	No OECD test guideline available
CYN (purity unknown)	Comet assay	HepG2 cells	12 h 24 h	0, 0.125, 0.25, and 0.5 µg/mL	Positive[o]	Štraser et al., 2013a	No OECD test guideline available
CYN (purity unknown)	Comet assay	HepG2 cells	4 h 24 h	0.01, 0.05, 0.1, 0.5 µg/mL	Positive[p]	Hercog et al., 2017	No OECD test guideline available
CYN purified from cyanobacterial cultures of *C. raciborskii Seenayya Subba Raju* (purity >98%)	Comet assay	Mouse hepatocytes	18 h	0.05, 0.1, 0.2, 0.5 µM, ± SKF525A or omeprazole.	Positive[q]	Humpage et al., 2005	No OECD test guideline available
CYN (purity unknown)	Double strand breaks	HepG2 cells	24 h 72 h	0, 0.125, 0.25 and 0.5 µg/mL	Positive[r]	Štraser et al., 2013b	No OECD test guideline available

Test article	Assay	Species	Time of exposure	Concentrations	Results	Reference	Remarks study protocol
	(γH2AX assay)						
CYN (purity unknown)	Double strand breaks (γH2AX assay)	HepG2 cells 3D model	72 h	0, 0.125, 0.25 and 0.5 µg/mL	Negative[s]	Hercog et al., 2020a	No OECD test guideline available
CYN (purity unknown)	Double strand breaks (γH2AX assay)	HepG2 cells	24 h 72 h	0 and 0.5 µg/mL	Positive[t]	Hercog et al., 2020b	No OECD test guideline available
CYN purified from cultures of *Raphidiopsis raciborskii* (purity unknown)	Double strand breaks (γH2AX assay)	HepaRG (undifferentiated)	24 h	0, 0.2 – 50 µM (0, 0.08 – 20.8 µg/mL)	Positive[u]	Huguet et al., 2019	No OECD test guideline available
Assays with extracts from bacterial blooms containing CYN							
Extract of bacterial bloom (CYN concentration 0.89 µg/L)	Bacterial reverse mutation (Ames) assay	*S. typhimurium* TA98 and TA100	90 min preincubation + 48 h	3.125 – 100% extract, equivalent to 0.03 – 0.89 ng/mL CYN, ± S9	Positive[v]	Sieroslawska, 2013	Ames microplate format mutagenicity assay.
Extract of bacterial bloom (CYN concentration 0.51 µg/L and MC-LR concentration 34.1 µg/L)	Bacterial reverse mutation (Ames) assay	*S. typhimurium* TA98 and TA100	90 min preincubation + 48 h	3.125 – 100% extract, equivalent to 0.02 – 0.51 ng/mL CYN and 1.07 – 34.1 ng/mL MC-LR, ± S9	Positive[w]	Sieroslawska, 2013	Ames microplate format mutagenicity assay.

CYN = cylindrospermopsin; DSB = double strand break; S9 = 9000 × g supernatant fraction from rat liver homogenate; MC-LR = microcystin-LR

[a] Three independent experiments were performed. Not reported whether preincubation method or direct plate method is used. No signs of precipitation of the test substance or toxicity to the bacterial strain were observed.

[b] Microplate format assay. Bacterial strains were incubated for 90 min with the test compound. Thereafter, the bacteria were diluted with pH indicator medium lacking histidine (for *S. typhimurium*) or tryptophan (for *E. coli*) and left in 384-well plates for the next two days at 37 °C. The number of wells containing revertant colonies was counted and the Fold Induction (FI) over the baseline was calculated. The baseline was obtained by adding one standard deviation to the mean number of positive wells of the controls. A fold induction > 2 is considered as a positive result. No signs of precipitation or of toxicity to the bacterial strain were observed.

[c] Cells were seeded at a density of 104 cells/mL in 96-well plates (two replicates per experimental group in each case). At the highest dose tested, the relative total growth compared to the control was 75 and 82 % (-S9, 4 h exposure), 37 and 29% (+S9, 4 h exposure) and 30 and 26% (-S9, 24 h exposure).

[d] After CYN exposure for 24 h, differentiated Caco-2 cells were treated with cytochalasin B (cytoB) for 29 h and thereafter had a 1.5 h recovery period. Undifferentiated Caco-2 cells were trypsinized, seeded and incubated for 48 h. Differentiated HepaRG cells were treated with cytoB for 44 h and thereafter had a 1.5 h recovery period and undifferentiated HepaRG cells were trypsinized, seeded and incubated for 24 h. Caco-2 cells: Statistically significant and dose-dependent increase in binucleated cells with micronuclei (MNBNC) frequency, to a greater extent in undifferentiated compared to differentiated cells. The highest dose(s) exceeded 50% cytotoxicity and cytotoxicity was higher in differentiated cells. No alterations in frequency of polynucleated, mitotic or apoptotic cells at any concentration. Mononucleated cells with micronuclei (MNMC) were also increased but this was not dose-dependent. HepaRG cells: dose-dependent decrease in hepatocyte-like cell number. MNBNC frequency statistically significantly increased in differentiated cells except at highest dose (most cytotoxic). No significant changes in frequency of mononucleated cells with MN, polynucleated, apoptotic or mitotic cells at any concentrations. After preincubation for 2 hr with ketoconazole, a CYP3A4 inhibitor, to a final concentration of 1, 5, or 10 µM, reduced micronucleus induction in Caco-2 cells, whereas in HepaRG cells no micronucleus induction was observed.

[e] Recovery time was respectively 18, 5 and 0 h. Colcemid was added during the last two h. Mitotic index was calculated counting 1000 cells per point. The analysis of chromosome aberrations (CA) was performed on a minimum of 100 metaphase cells per point. No increase in micronucleus frequency was observed. The mitotic index values decreased in a dose- and time-dependent manner.

[f] Cells were exposed for 24 h to CYN in the presence of cytoB (4.5 µg/mL), with a 24 h recovery period (in the presence of cytoB as well). Positive at the highest concentration group. The frequency of micronuclea was statistically significantly higher in the highest treatment group (an average fold induction of 2.3. The vast majority of detected micronuclei was smaller than ¼ the size of normal nuclei. The nuclear division index (NDI) was decreased at the highest CYN concentration.

[g] After exposure to CYN, cells were exposed to cytoB (6 µg/mL) for 20 h. Micronuclei frequencies were scored in at least 2000 binucleated cells per concentration. The frequency of binucleated cells with micronuclei (BNMN) and the cytokinesis-block proliferation index (CBPI) were analyzed according to the recommendations of OECD 487 (2016). A statistically significant increase in the frequency of BNMN was observed between 0.25 and 2.0 µg/mL in the presence of S9. No significant variations were observed in CPBI.

[h] Three independent experiments were performed. After exposure to CYN (±MC-LR), cells were incubated for 26 h in the presence of cytoB. Micronuclei were counted in 1000 binucleated cells (BNC) per experimental point. The NDI was estimated by scoring 500 cells. At the highest concentration, CYN statistically significantly induced the micronucleus frequency (>2-fold). The NDI was statistically significantly reduced at this concentration. The CYN/MC-LR mixture induced the same responses as CYN alone.

[i] Micronuclei, nuclear buds and nuclear bridges (NPBs) were counted in 1,000 binucleated cells (BNC). Statistically significant increases in the frequencies of micronuclei and micronucleated cells was detected at 0.05 and 0.5 µg/mL CYN The frequency of nuclear buds was statistically significantly increased at 0.05 and 0.5 µg/mL. An increase in nuclear bridges frequency was detected at 0.05 and 0.5 µg/mL but reached statistical significance only at 0.05 µg/mL CYN. A statistically significant decrease in NDI was determined in cells exposed to 0.5 µg/mL of CYN.

[j] For each experiment 100 cells per concentration were analyzed and two or three independent experiments were performed. Cell viability was not affected, whereas growth inhibiton (>50%) was observed at both test concentrations.

[k] Four independent experiments were performed and at least 100 images per dose and treatment time were analyzed. The %tail DNA was statistically significantly increased at 150 µg/L (after 48 and 72h exposure) and 300 µg/L (after all exposure durations).

[l] Three independent experiments were performed. Negative results were obtained in both the standard comet assay as well as in the enzyme modified comet assay using the enzymes Endonuclease III and Formamido-pyrimidine DNA glycosylase, which detect oxidized bases. The test concentrations were based on a previous cytotoxicity test, but no actual cytotoxicity data for this study were provided.

[m] Two slides were prepared for each treatment. The %tail DNA was determined for at least 100 nuclei per treatment and per control, and was statistically significantly higher in the treated group compared to the control group.

[n] Three independent experiments were performed and 50 nuclei were analyzed per treatment. Statistically significant increase in DNA damage was detected after 12 h and 24 h exposure to 0.5 µg/ml and 0.01, 0.05, 0.1 and 0.5 µg/ml CYN respectively, but not after 4 h exposure.

[o] Three independent experiments were performed and 50 nuclei were analyzed per treatment. Statistically significant increase in DNA strand breaks and oxidative DNA damage was detected at the highest two concentrations of CYN (0.25 and 0.5 µg/ mL) after 12 h and 24 h exposure but not after 4 h exposure. The DNA damage caused by CYN was unlikely to be due to oxidative stress.

[p] Three independent experiments were performed wherein fifty randomly selected nuclei were analyzed per experimental point. After 4 h exposure only MC-LR (1 mg/mL) induced statistically significant increase of DNA strand breaks. After 24 h exposure, CYN induced statistically significant increase in the amount of DNA strand breaks at concentrations ≥0.05 mg/mL. The levels of DNA strand breaks induced by the MC-LR/CYN mixtures were lower from those induced by CYN alone and were not statistically significantly different from the background level.

[q] Between 40 and 50 comets were scored per slide. For each concentration, three different slides were scored (i.e., 120 to 150 comets). CYN clearly produced significant DNA fragmentation at concentrations as low as 0.05 µM. In some instances, cells were preincubated for approximately 30 to 45 min with SKF525A or omeprazole at final concentrations of 50 and 100 µM, respectively, prior to the addition of CYN. Both SKF525A and omeprazole both prevented the increase in comet tail lengths, areas, and moments.

[r] Three independent experiments were performed. The presence of double strand breaks (DSBs) was analyzed by flow cytometry, indirectly through the detection of γH2AX foci. After 72 h of exposure, DSBs were statistically significantly induced at 0.5 µg/mL. At 0.125 µg/mL (after 24 h and 72 h) and 0.25 µg/mL (after 72 h), DSBs were statistically significantly reduced compared to the control, possibly due to DSB repair processes or the protein synthesis inhibition.

[s] Three independent experiments were performed. No statistically significant increase in induction of γH2AX formation was observed.

[t] Three independent experiments were performed. A statistically significant increase in induction of γH2AX formation was observed after 72 but not after 24 h.

[u] Three independent experiments were performed. A dose dependent increase (statistically significant from 12.5 µM onwards) in γH2AX positive cells was observed.

[v] Positive results were obtained for strain TA98 +S9 and strain TA100 ±S9. No signs of precipitation or of toxicity to the bacterial strain were observed.

[w] Positive results were obtained for strain TA100 ± S9. No signs of precipitation or of toxicity to the bacterial strain were observed.

Annex 2. In vivo genotoxicity tests with cylindrospermopsin (CYN)

Test article	**Assay**	**Species (tissues analysed)**	**Dose and route**	**Toxicity**	**Result assay**	**Reference**
CYN (purity 98%)	Micronucleus assay	Swiss albino mice, male (colon crypts)	0, 1, 2 and 4 mg/kg bw (single dose; p.o. gavage)	Systemic toxicity	Negative[a]	Bazin et al. 2012
CYN (purity 98%)	Micronucleus assay	Swiss albino mice, male (bone marrow)	0, 50, 100 and 200 µg/kg bw (single dose; i.p.)	Systemic toxicity	Negative[b]	Bazin et al. 2012
CYN (purity 95%)	Micronucleus assay	Wistar rats, male (bone marrow)	0, 7.5, 23.7 and 75 µg/kg bw (three doses at 0, 24 and 45 h; p.o. gavage)	Cytotoxicity at 75 µg/kg bw	Positive[c]	Diez-Quijada et al. 2019
CYN (purity 95%) + MC-LR (purity 99%)	Micronucleus assay	Wistar rats, male + female (bone marrow)	0, 7.5 CYN + 75 MC-LR, 23.7 CYN + 237 MC-LR and 75 CYN + 750 MC-LR µg/kg bw (three doses at 0, 24 and 45 h; p.o. gavage)	Cytotoxicity at 75 µg/kg bw	Positive[d]	Diez-Quijada et al. 2020
CYN (purity 98%)	Comet assay	Swiss albino mice, male (blood, bone marrow, kidney, liver, ileum and colon)	0, 1, 2 and 4 mg/kg bw (single dose; p.o. gavage)	Systemic toxicity	Positive[e]	Bazin et al. 2012
CYN (purity 98%)	Comet assay	Swiss albino mice, male (blood, bone marrow, kidney, liver, ileum and colon)	0, 50, 100 and 200 µg/kg bw (single dose; i.p)	Systemic toxicity	Positive[f]	Bazin et al. 2012
CYN (purity 95%)	Comet assay	Wistar rats, male	0, 7.5, 23.7 and 75 µg/kg bw (three doses at 0, 24 and 45 h; p.o. gavage)	Cytotoxicity at 75 µg/kg bw	Negative[g]	Diez-Quijada et al. 2019

		(blood, liver and stomach)				
CYN (purity 95%) + MC-LR (purity 99%)	Comet assay (standard assay and enzyme-modified assay)	Wistar rats, male + female (blood, liver and stomach)	0, 7.5 CYN + 75 MC-LR, 23.7 CYN + 237 MC-LR and 75 CYN + 750 MC-LR µg/kg bw (three doses at 0, 24 and 45 h; p.o. gavage)	Cytotoxicity at 75 µg/kg bw	Negative[h]	Diez-Quijada et al. 2020
CYN (purity 98%)	Comet assay	Albino Wistar rats, male (liver)	0, 79.80 µg/kg bw (single dose, i.p.)	Systemic toxicity	Positive[i]	Dordevic et al. 2017
Methanolic extract of *C. raciborskii* containing 6.65 µg CYN/mg extract	Comet assay	Albino Wistar rats, male (liver)	0, 1500, 3000, 6000 and 12,000 µg/kg bw corresponding to 0, 9.97, 19.95, 39.90 and 79.80 µg CYN/kg bw, respectively (single dose; i.p.)	Systemic toxicity	Positive[j]	Dordevic et al. 2017
CYN (extracted and purified from a culture of *C. Raciborskii*, purity not reported)	DNA strand breaks	Balb/c mice (liver)	0.2 mg/kg bw CYN (single dose; i.p.)	Not reported	Positive[k]	Shen et al., 2002
Cell-free extract of *C. Raciborskii*	DNA adducts (^{32}P postlabelling assay)	White Quackenbush mice, male (liver	Unknown (single dose, i.p.)	Not reported	Positive[l]	Shaw et al., 2000

i.p. = intraperitoneal; p.o. = per os; MC-LR = microcystin-LR

[a] Part of combined Comet/micronucleus assay. Animals were killed 24 h after administration. Intact colon crypts were chosen for scoring at least 1000 cells per mouse. One mouse receiving an oral dose of 4 mg/kg bw was moribund after 24 hours and examination revealed a dark red liver and intestinal haemorrhage and another mouse had an

intestinal bleeding and liquid stool. One mouse of the 2 mg/kg bw dose group died. Histological analysis showed apoptosis in both liver and kidney tissues at 2 and 4 mg/kg bw, and in some mice, apoptosis of lymphocytes within Peyer's patches in duodenum and jejunum.

[b] Part of combined Comet/micronucleus assay. Animals were killed 24 h after administration. Two thousand polychromatic erythrocytes and at least 1000 NCE per animal were analysed for micronuclei. No mortality was observed; one mouse which received highest dose was observed to have a pale liver. Histological examination revealed cell death in both liver and kidney at 100 and 200 µg/kg bw. In accordance with OECD guideline 474.

[c] Part of combined Comet/micronucleus assay. Animals were killed 3 h after the last administration and bone marrow samples were analysed. Histological examination revealed mucosal damage and modifications in the nuclei structure of liver cells and proliferation of smooth endoplasmic reticulum in treated rats and at the highest dose a high mitotic frequency. Statistically significant increases in %MN were observed for all dose groups, but no dose-dependency was observed. Statistically significant decreases in PCE:total erythrocytes and PCE:NCE ratios were observed at 75 µg/kg bw. In accordance with OECD guideline 474.

[d] Part of combined Comet/micronucleus assay. Animals were killed 3 h after the last administration and bone marrow samples were analysed. In the high dose group, degenerate and necrotic hepatocytes in centrilobular areas were observed together with intracellular lipid accumulation in hepatocytes (macro vesicular vacuolation). In addition, multinuclear hepatocytes were present. Statistically significant increases in %MN in immature erythrocytes observed at all doses in both sexes compared to controls. Statistically significant decreases in PCE:total erythrocytes and PCE:NCE ratios observed in both sexes at highest dose compared to controls. In accordance with OECD guideline 474.

[e] Part of combined Comet/micronucleus assay. Animals were killed 24 h after administration. One mouse receiving an oral dose of 4 mg/kg bw was moribund after 24 hours and examination revealed a dark red liver and intestinal haemorrhage and another mouse had an intestinal bleeding and liquid stool. One mouse of the 2 mg/kg bw dose group died. Histological analysis showed apoptosis in both liver and kidney tissues at 2 and 4 mg/kg bw, and in some mice, apoptosis of lymphocytes within Peyer's patches in duodenum and jejunum. At 1 and 2 mg/kg bw, a statistically significant increase in %tail DNA in bone marrow cells (28.7% and 20%, respectively, compared to approximately 16% in the controls) was observed and at 4 mg/kg bw in colon cells (38% compared to approximately 20% in the controls). At 4 mg/kg, no significant increase in %tail DNA in bone marrow was observed, but there was a high variability between mice (median 22% increase).

[f] Part of combined Comet/micronucleus assay. Animals were killed 24 h after administration. No mortality was observed; one mouse which received highest dose was observed to have a pale liver. Histological examination revealed cell death in both liver and kidney at 100 and 200 µg/kg bw. At 100 and 200 µg/kg bw, a statistically significant increase in %tail DNA (24.7% and 12.2%, respectively, compared to 8.5% in the controls) was observed in the colon.

[g] Part of combined Comet/micronucleus assay. Animals were killed 3 h after the last administration. Histological examination revealed mucosal damage and modifications in the nuclei structure of liver cells and proliferation of smooth endoplasmic reticulum in treated rats and at the highest dose a high mitotic frequency. In accordance with OECD guideline 489.

[h] Part of combined Comet/micronucleus assay. Animals were killed 3 h after the last administration. In the enzyme-modified Comet assay, the slides were treated with the enzymes Endonuclease III (EndoIII) and Formamidopyrimidine DNA glycosylase (Fpg) before analysis to detect oxidative DNA damage. In the high dose group, degenerate and necrotic hepatocytes in centrilobular areas were observed together with intracellular lipid accumulation in hepatocytes (macro vesicular vacuolation). In addition, multinuclear hepatocytes were present. In accordance with OECD guideline 489.

[i] Data from three independent experiments were reported. Animals were killed 24 or 72 h after treatment and the livers were analysed. Histopathological and morphological examination showed liver damage (sinusoidal dilatation of central vein, hydropic degeneration, intralobular infiltration, focal lytic necrosis). Statistically significant increases were observed in all three parameters analysed (tail length, %DNA in tail and tail moment) compared to negative control at both timepoints.

[j] Data from three independent experiments were reported. Animals were killed 24 or 72 h after treatment and the livers were analysed. Histopathological and morphological examination showed liver damage (sinusoidal dilatation, hydropic degeneration, intralobular infiltration, focal lytic necrosis, vacuolar degeneration, apoptosis, Kupferr cell hypertrophy), that was most severe 72 h after treatment with 3000 or 6000 µg/kg bw. Statistically significant increases were observed in all three parameters analysed (tail length, %DNA in tail and tail moment) compared to negative control at all doses at both timepoints. The increases were generally dose-related.