Literature review of the toxicity of naturally occurring cyanotoxins of drinking water relevance

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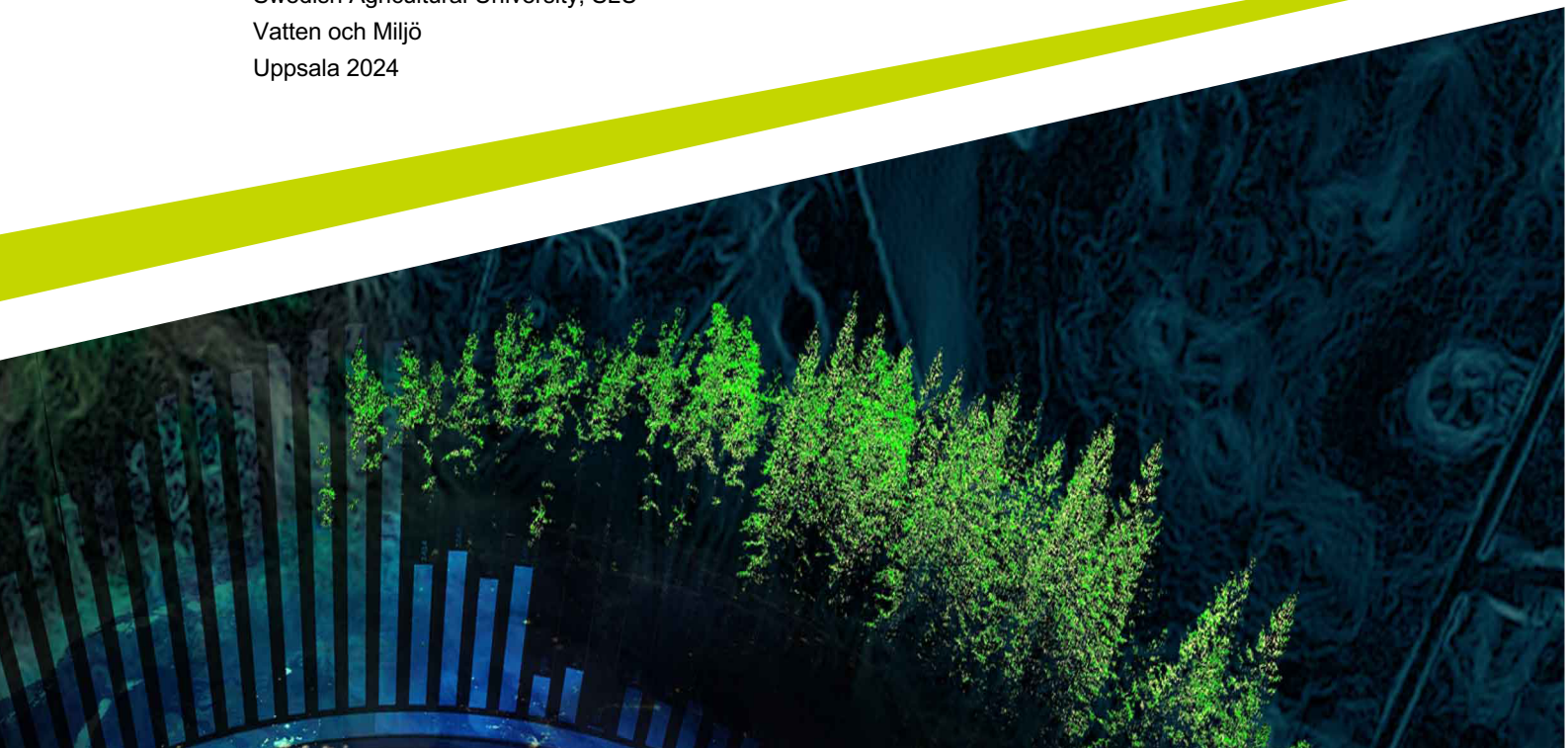

Literature review of the toxicity of naturally occurring cyanotoxins of drinking water relevance.

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Abstract

This paper provides a comprehensive literature review focusing on the genotoxic, oxidative stress, and neurotoxic effects of two cyanotoxins, cylindrospermopsin (CYN) and anatoxin (ATX), commonly found in freshwater reservoirs worldwide. The research addresses the growing concern of cyanobacteria presence in drinking water, driven by factors like global warming, urbanization, and sewage discharges. Given the cyanobacteria's resistance to conventional purification methods, the importance of this report is emphasized.

By utilizing the Scopus database, the study collects relevant information and examines the toxicological profiles of CYN and ATX, aiming to understand their varying potencies. The selection of relevant studies is based on predefined search criteria, focusing on in vitro and in vivo experiments with pure toxin samples. To facilitate comparisons, LOEC (Lowest Observed Effect Concentration) and NOEC (No Observed Effect Concentration) values are determined, and unit conversions are performed for consistency.

Results indicate that oxidative stress appears to be the most sensitive toxicity parameter, followed by genotoxicity and neurotoxicity. However, comparisons between CYN and ATX reveal nuanced differences in potency, with CYN showing higher potency for oxidative stress and genotoxicity based on available data. The methods used for assessing these toxicities demonstrate reliability, particularly techniques measuring ROS, DNA damage, and AChE activity.

Despite the strengths of molecular assessments, the paper highlights the limitations of solely relying on them to predict broader physiological impacts in humans. Combining in vivo studies with molecular assessments offers a more comprehensive understanding of how CYN and ATX could affect human health. Moreover, there is an imperative need for cost-effective techniques to combat cyanotoxins in water treatment. Overall, the findings underscore the importance of ongoing research to better understand and mitigate the risks associated with cyanotoxin contamination in drinking water.

Keywords: cylindrospermopsin, anatoxin, genotoxicity, oxidative stress, neurotoxicity, drinking water

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Abbreviations

ATX	Anatoxin
CYN	Cylindrospermopsin
LOEC	Lowest Observed Effect Concentration
NOEC	No Observed Effect Concentration
ROS	Reactive oxygen species
RONS	Rective oxygen and nitrogen species
CAT	Catalase
GSH	Glutathione
GSSG	Glutathione disulfide ratio
GST	Glutathione S-transferase
MDA	Malondialdehyde
SOD	Superoxide dismutase
GPx	Glutathione peroxidase
LPO	Lipid peroxidation
MNI	Micronuclei
NPB	Nucleoplasmic bridges
NBUDs	Nuclear buds
AChE	Acetylecholinesterase
CBMN	Cytokinesis-block micronucleus assay
PAC	Powdered activated carbon
GAC	Granular activated carbon

1. Introduction

Hidden within the waterbodies of our beloved planet lurks many, almost invisible, threats. Despite their microscopic reveal they are powerful enough to pose a significant concern to human health. One such concealed agent is the cyanobacteria, a blue-green algae that produce a group of toxins called cyanotoxins. As these toxins can find their way into our bodies through various routes, drinking contaminated water, is the primary source of exposure. Cyanotoxins produced by the algae are so-called secondary metabolites, and two such emerging cyanotoxins are cylindrospermopsin (CYN) and anatoxin (ATX). The two substances are produced by several cyanobacterial species and naturally dissolve very well in water (Plata-Calzado et al. 2022).

The toxicity of cylindrospermopsin and anatoxin has been explored through both experiments in cells outside living organisms (in vitro) and within living organisms (in vivo), uncovering various effects. One of the primary benefits of in vitro studies is the high degree of control they provide over the experimental environment as they are conducted in a controlled environment, such as in petri dishes or test tubes. However, in vitro experiments have limitations as well. They lack the complex interactions present in whole organisms, which can lead to results that do not fully translate to in vivo situations. Moreover, cultured cells used in these experiments can differ significantly from their in vivo counterparts, potentially affecting the validity of the results. In contrast, in vivo experiments involve studying the effects of substances within a living organism, providing a more comprehensive and realistic assessment of biological responses. One significant advantage of in vivo studies is their ability to offer insights into how toxins interact with various biological barriers, such as the digestive system, bloodstream, and blood-brain barrier. Additionally, they enable the study of drug absorption, distribution, metabolism, and excretion (ADME) within a living system, providing critical information on how a substance behaves within an organism. On the other hand, these studies are also more expensive and biological variability between individual animals can complicate data interpretation and often requires larger sample sizes to achieve statistical significance.

One toxicity that is commonly investigated using in vitro and in vivo studies is oxidative stress. The mechanism behind the toxic effect is the alterations of certain antioxidant measures, for example; catalase (CAT) and glutathione (GSH) (Zhong

et al. 2020). In a study on rat liver cells, so-called hepatocytes, conducted by Maria T. Runnegar et al. (1995), showed that the production of GSH, which is one of the major antioxidants produced in a mammal body, were inhibited by CYN. Therefore, the decrease of GSH caused by CYN, plays an important role in the induction of oxidative damage (Runnegar et al. 1995). Similar to how CYN alter the activity of GSH, ATX can cause an increase in CAT level which, as mentioned earlier, is a part of the cell's antioxidant defense mechanisms. This was proven in a study by Mitrovic et al. (2004), and it suggests that the generation of reactive oxygen species within the cell triggers a protective response and therefore an increase of CAT.

Another toxic effect that has been investigated by researchers is cytotoxicity, which is the ability of a substance to cause damage to cells and decrease their viability. Genotoxicity is another toxicity parameter that targets the genes of the cells. One type of genotoxicity is mutagenicity, which is the property of damaging the genes by causing mutations. However, this report will not focus on either mutagenicity or cytotoxicity. On the other hand, this report will focus on genotoxicity where cyanotoxins can cause breakage of DNA strands or alter the processes responsible for maintaining DNA integrity. Consequently, techniques to detect the genotoxic effect is to measure DNA damage. The techniques for this kind of measurement are called the standard comet assay or the enzyme modified comet assay (Puerto et al. 2018). As mentioned earlier, genotoxicity can also imply disturbance of cellular processes that preserve the DNA. For example, the outcome of such toxic effect is the formation of so-called micronuclei which are small, extra nuclei that can form if cellular processes handling DNA is not properly working. Instead of using the standard comet assay, the method appropriate for detecting this kind of DNA damage is called cytokinesis-block micronucleus assay (CBMN) (Sieroslawska and Rymuszka 2015).

Neurotoxicity may also be an adverse effect caused by cyanotoxins. For example, ATX and CYN can cross the blood-brain barrier in fish and directly interfere with neurological functions. In this case, one key mechanism involves the alteration of the neurotransmitter acetylcholine (ACh), particularly its degrading enzyme acetylcholinesterase (AChE). Thus, by measuring the activity of AChE following a cyanotoxin exposure, it can indicate a potential neurotoxic effect (Plata-Calzado et al. 2022).

In evaluating the toxicity of substances such as CYN and ATX, a common way is to observe the lowest concentration where effect was induced, and to determine the highest concentration, for when no effect was induced. The concepts are called Lowest Observed Effect Concentration, or LOEC, and No Observed Effect Concentration, or NOEC. By determining LOEC and NOEC for several studies concerning for example anatoxin, one can assess thresholds at which anatoxin can cause a toxic effect. The thresholds or the different LOEC's can be used for further analyzation. One such analysis can be comparisons between the sensitivity of

different parameters such as neurotoxicity or genotoxicity. In simpler terms, it helps to determine whether anatoxin is more likely to cause genotoxic effects than neurotoxic effects.

The two cyanotoxins with their concerning characteristics, have been found in water sources worldwide. Germany being one example, where ATX was discovered in several lakes with the highest concentration of 13.1 µg/L (Testai et al. 2016). Moreover, a cyanobacterial bloom in Lake Taihu in China disrupted the water supply for two million individuals, resulting in a lack of access to drinking water for more than a week (Kumar et al. 2018). The effects of cyanotoxins on humans are not well known, but a notable example is the incident in Caruaru, Brazil, in February 1996. During this event, cyanotoxins caused acute liver failure in 101 out of 130 hemodialysis patients, resulting in 50 deaths by September 15, 1996. The cause was identified as cyanobacterial contamination of the water used for dialysis (Jochimsen Elise M. et al. 1998).

As different cyanotoxins vary in their molecular size, structure, and reactivity with disinfectants, for example anatoxins which shows resistance to chlorination, traditional water treatment methods are often inadequate in eliminating cyanotoxins (Himberg et al. 1989; Almuhtaram et al. 2018). However, innovative treatments, such as pre-ozonation combined with powdered activated carbon or PAC (Maatouk et al. 2002) and granular activated carbon (GAC) (He et al. 2016; Marsálek et al. 2005a) have shown promise in removing the toxins under specific conditions. Current research is also exploring modified GAC techniques for improved removal of various organic compounds (Crowe et al. 2022). Other evaluated techniques are various membranes including nanofiltration membrane NF-270 and sulfonated polysulfone membranes TRISEP, HYDRACoRe-10, and HYDRACoRe-50, in removing anatoxin and cylindrospermopsin (Lebad et al. 2024). Additionally, a water treatment process called bank filtration has demonstrated effectiveness in reducing cyanotoxins (Walkenhorst et al. 2021).

According to the Swedish Food Agency, action should be taken when the level of ATX in drinking water reaches 7.5 µg/L. This guideline value is calculated from the temporary acute reference dose (tARfD) of 1.25 micrograms of anatoxin per kilogram of body weight per day and under the assumption that 100% of the ATX intake is allocated to drinking water. Similarly, the Agency establishes a safety benchmark for cylindrospermopsin, with a temporary reference dose of 0.1 micrograms per kilogram of body weight per day. This sets a guideline value of 0.6 micrograms of cylindrospermopsin per liter of drinking water for when actions may need to be taken. However, it's crucial to note that there are significant uncertainties associated with these values (*Livsmedelsverkets* u.å.).

As conventional water treatment methods are ineffective in completely removing cyanotoxins (Himberg et al. 1989), the importance of this report stems from the critical issue that cyanobacteria are increasingly present in drinking water,

and this trend is expected to continue due to ongoing global warming, urbanization, and sewage discharges (Paerl & Huisman 2009). While the individual effects of anatoxin and cylindrospermopsin are the primary focus in this report, it is important to consider their combined impact, as organisms are typically exposed to several toxins simultaneously; however, this report will not address this combined "cocktail" effect.

This report conducts a thorough literature review, delving into existing research to analyze the genotoxic, oxidative stress, and neurotoxic effects of the cyanotoxins cylindrospermopsin and anatoxin. Given the significance of this research, which stems from the widespread presence of CYN and ATX in drinking water, the primary objective is to understand and compare the toxicological profiles of these toxins and to elucidate their varying potencies.

2. Method

For the collection of sources and information upon which this study is built, the database Scopus has been utilized and the results from the selected keywords below have been employed. Initially, 'cylindrospermopsin', ‘anatoxin’ and 'toxicity' were queried, resulting in over a thousand results. To refine the search and narrow down the findings, the word ‘human’ was added, however the results remained too many to thoroughly investigate. Consequently, toxicities such as genotoxicity and oxidative stress were selected based on a quick overview of the documents, indicating their relevance. Therefore, the next selected thread for further investigation was ‘cylindrospermopsin’, ‘genotoxicity’ and ‘oxidative stress’ which resulted in 24 documents. The thread ‘anatoxin’, ‘genotoxicity’ and ‘oxidative stress’ resulted in 6 documents. This number of results, 24 and 6, were considered adequate for in-depth reading and are for that reason the foundation of the study.

Additionally, the relationship between the toxins and neurotoxicity was investigated. The reason was the poor amount of toxicity studies on anatoxin, and after some reading, neurotoxicity was decided to be the most adequate parameter to add. When ‘neurotoxicity’ was added to the thread it resulted in 8 and 13 documents (for ATX and CYN respectively). The final threads that were used and are the base for the entire study are:

‘cylindrospermopsin AND genotoxicity AND "oxidative stress"’; ‘anatoxin AND genotoxicity AND "oxidative stress"’; ‘cylindrospermopsin AND neurotoxicity AND (“oxidative stress” OR genotoxicity)’; ‘anatoxin AND neurotoxicity AND (“oxidative stress” OR genotoxicity)’

When analyze current water treatment for anatoxin and cylindrospermopsin the following thread was used:

‘“drinking water” AND (detoxification OR purification OR removal) AND cylindrospermopsin AND anatoxin’

To investigate the effects of the cyanotoxins, the initial step was to review the studies included in the total of 51 reports that resulted from the search threads. As the in-depth reading of the reports had been initiated, it was decided to only include experiments conducted with pure toxin rather than extracts of cyanobacteria. The cause was to narrow down the analysis and to simplify comparison between the different experiments. Additionally, it became evident that investigating all

available studies, both reviews and individual experiments, would have been an extensive task. To analyze all the sources referenced in all the reviews would have been too time consuming, considering the length of this project. Although some references from a couple reviews were included, a complete analysis of all reviews and their containing references were not conducted. Instead, the emphasis was placed on the individual studies.

To compare different concentrations where toxic effect was induced by CYN and ATX, a LOEC and a NOEC was determined for all reports with sufficient results. However, the reports analyzed did not explicitly provide LOEC and NOEC values for the studied toxins. Therefore, through careful examination of the presented data, including diagrams and textual descriptions, LOEC and NOEC values were determined. These values were then used for the comparative analysis of the toxins' toxicity profiles. For making the comparison of the result easier, average values were calculated from the different Lowest Observed Effect Concentrations for each unit and also, respectively for the in vitro and in vivo studies.

Moreover, while writing the result, conversion of units needed to be done, to ease inter-studies comparisons. To convert the concentration of cylindrospermopsin and anatoxin from micromole per liter (μM) to micrograms per liter (μg/L), the molecular mass of CYN (415.4 g/mol) and ATX (281.3 g/mol) was used. The calculation was performed by multiplying the given concentration in micromoles per liter by the molecular mass, according to the formula: Concentration (μg/L) = Concentration (μM) x Molecular mass (g/mol).

3. Results

3.1 Data collection of studies

Table 1. Summary of studies investigating the toxicity of anatoxin and cylindrospermopsin

Study	Toxin	Type of toxicity	Model system	Variable measured	Interval of concentrations	LOEC/NOEC
(Mitrovic et al. 2004)	ATX	Oxidative stress	In vivo, plant species L. minor (duckweed)	Activity of peroxidase (POD)	5, 15, and 25 µg mL in water 4 days	LOEC: 25 µg/mL NOEC: 15 µg/mL
(Mitrovic et al. 2004)	ATX	Oxidative stress	In vivo, plant species L. minor (duckweed)	Activity of soluble glutathione S-transferases (cGST)	0,1 to 20 µg/mL in water 24 h	LOEC: 5.0 µg/mL NOEC: 1.0 µg/mL
(Mitrovic et al. 2004)	ATX	Oxidative stress	In vivo, plant species L. minor (duckweed)	Activity of catalase (CAT)	0,1 to 20 µg mL in water 24 h	LOEC: 5.0 µg/mL NOEC: 1.0 µg/mL.

(Mitrovic et al. 2004)	ATX	Oxidative stress	In vivo, plant species C. fracta (water fern)	Activity of peroxidase (POD)	10, 15, and 25 µg mL in water 4 days	LOEC: 25 µg/mL NOEC: 15 µg/mL
(Zhong et al. 2020)	ATX	Oxidative stress	In vitro, carp immunocytes	ROS content	0.01, 0.1, 1, and 10 mg/L	LOEC: 0.01 mg/L*
(Zhong et al. 2020)	ATX	Oxidative stress	In vitro, carp immunocytes	MDA content	0.01, 0.1, 1, and 10 mg/L	LOEC: 0.1 mg/L NOEC: 0.01 mg/L
(Zhong et al. 2020)	ATX	Oxidative stress	In vitro, carp immunocytes	SOD content	0.01, 0.1, 1, and 10 mg/L	LOEC: 0.01 mg/L*
(Zhong et al. 2020)	ATX	Oxidative stress	In vitro, carp immunocytes	GPx activity	0.01, 0.1, 1, and 10 mg/L	LOEC: 0.01 mg/L*
(Sierosławska & Rymuszka 2013)	ATX	Genotoxicity	In vitro, carp leukocytes	DNA damage	0.5 µg/mL	No observed effect
(Osswald et al. 2011)	ATX	Neurotoxicity	In vivo, rainbow trout (Oncorhynchus mykiss)	Behavioral differences	132, 264 and 524 µg/L in water	LOEC: 132 µg/L* (0.132 µg/mL)

(Osswald et al. 2013)	ATX	Neurotoxicity	In vivo, rainbow trout (Oncorhynchus mykiss)	Muscle AChE activity	0.08, 0.12, 0.20, 0.31 µg/g body weight in fish	LOEC: 0.20 µg/g NOEC: 0.12 µg/g
(Osswald et al. 2013)	ATX	Oxidative stress	In vivo, rainbow trout (Oncorhynchus mykiss)	GST level	0.08, 0.12, 0.20, 0.31 µg/g body weight in fish	LOEC: 0.31 µg/g NOEC: 0.2 µg/g
(Lakshmana Rao et al. 2002)	ATX	Genotoxicity	In vitro, rat immune cells called thymocytes	DNA fragmentation	1 – 10 µg/mL	LOEC: 4 µg/mL NOEC: 2 µg/mL
(Rabelo et al. 2021)	CYN	Genotoxicity and neurotoxicity	In vivo, female fish Pecilia reticulata	DNA damage, in the brain	0.5, 1.0, and 1.5 µg/L in water 24 and 92 h	LOEC: After 24 h 1.0 µg/L After 92 h* 0.5 µg/L

						NOEC: After 24 h 0.5 µg/L
(Takser et al. 2016)	CYN	Neurotoxicity	In vitro, N2a or Neuro 2A a mouse neurblastoma-derived cell line	Viability of the neuroblastoma	0.4154, 41.54 and 4154 µg/L 24 h	LOEC: 41.54 µg/L NOEC: 0.4154 µg/L
(Takser et al. 2016)	ATX	Neurotoxicity	In vitro, N2a or Neuro 2A a mouse neurblastoma-derived cell line	Viability of the neuroblastoma	0.2813, 28.13 and 2813 µg/L 24 h	LOEC: 28.13 µg/L NOEC: 0.2813 µg/L
(Takser et al. 2016)	CYN	Neurotoxicity	In vitro, N2a or Neuro 2A a mouse neurblastoma-derived cell line	Activity of enzymes involved in apoptosis	0.4154, 41.54 and 4154 µg/L 24 h	LOEC: 4154 µg/L NOEC: 41.54 µg/L

(Takser et al. 2016)	ATX	Neurotoxicity	In vitro, N2a or Neuro 2A a mouse neurblastoma-derived cell line	Activity of enzymes involved in apoptosis	0.2813, 28.13 and 2813 µg/L 24 h	LOEC: 2813 µg/L NOEC: 28.13 µg/L
(Hinojosa et al. 2019)	CYN	Neurotoxicity	In vitro, immature SH-SY5Y cells derived human neuroblastoma	Activity of acetylcholinesterase	0-1 µg/mL	LOEC: No observed effect
(Hinojosa et al. 2019)	CYN	Neurotoxicity	In vitro, mature SH-SY5Y cells derived human neuroblastoma	Activity of acetylcholinesterase	0–0.3 µg/mL	LOEC: 0.075 µg/mL*
(Gutiérrez-Praena et al. 2012b)	CYN	Oxidative stress	In vivo, male fish O. niloticus	Level of lipid peroxidation (LPO) in the liver and kidney	200 µg/kg in feed	LOEC: Effect observed in liver and kidney*
(Gutiérrez-Praena et al. 2012b)	CYN	Oxidative stress	In vivo, male fish O. niloticus	Level of protein carbonyl content	200 µg/kg in feed	LOEC: In liver, no observed effect In kidney, observed effect*
(Gutiérrez-Praena et al. 2012b)	CYN	Oxidative stress	In vivo, male fish O. niloticus	Glutathione and glutathione disulfide ratio (GSH/GSSG) levels	200 µg/kg in feed	LOEC: In liver, observed effect*

						In kidney, no effect
(Gutiérrez-Praena et al. 2012b)	CYN	Oxidative stress	In vivo, male fish O. niloticus	Levels of γ-glutamylcysteine synthetase level (GCS)	200 µg/kg in feed	LOEC: In liver, observed effect* In kidney, observed effect*
(Gutiérrez-Praena et al. 2012b)	CYN	Oxidative stress	In vivo, male fish O. niloticus	Level of glutathione S-transferase (GST)	200 µg/kg in feed	LOEC: In liver, effect observed* In kidney, no observed effect
(Gutiérrez-Praena et al. 2012b)	CYN	Oxidative stress	In vivo, male fish O. niloticus	Measuring GPx activity	200 µg/kg in feed	No effect in kidney and liver
(Liebel et al. 2011)	CYN	Oxidative stress	In vitro, hepatocytes from fish P. lineatus.	Level of RONS (reactive oxygen/nitrogen species)	0.1, 1.0, 10 µg/L	LOEC: 0.1 µg/L*
(Liebel et al. 2011)	CYN	Oxidative stress	In vitro, hepatocytes from fish P. lineatus.	Glutathione S-transferase (GST) activity	0.1, 1.0, 10 µg/L	LOEC: 1.0 µg/L

						NOEC: 0.1 µg/L
(Liebel et al. 2011)	CYN	Oxidative stress	In vitro, hepatocytes from fish P. lineatus.	Glucose-6-phosphate dehydrogenase (G6DH) activity	0.1, 1.0, 10 µg/L	LOEC: 0.1 µg/L*
(Liebel et al. 2011)	CYN	Oxidative stress	In vitro, hepatocytes from fish P. lineatus.	Glutathione (2GSH/GSSG) ratio	0.1, 1.0, 10 µg/L	No observed effect
(Liebel et al. 2011)	CYN	Oxidative stress	In vitro, hepatocytes from fish P. lineatus.	Protein carbonylation (PCO) level	0.1, 1.0, 10 µg/L	No observed effect
(Liebel et al. 2011)	CYN	Oxidative stress	In vitro, hepatocytes from fish P. lineatus.	Lipid peroxidation (LPO)	0.1, 1.0, 10 µg/L	LOEC: 10 µg /L NOEC: 1 µg/L
(Liebel et al. 2011)	CYN	Genotoxicity	In vitro, hepatocytes from fish P. lineatus.	DNA damage	0.1, 1.0, 10 µg/L	No observed effect
(Colas et al. 2020)	ATX	Neurotoxicity	In vivo, medaka fish	Behavioral differences	1.1 – 20 µg/g body weight in fish	LOEC: 20 µg/g

						NOEC: 6.67 µg/g
(Guzmán-Guillén et al. 2015)	CYN	Oxidative stress	In vivo, O. niloticus (Nile tilapia)	Lipid peroxidation (LPO)	42.4 µg/L in water	LOEC: Effect observed*
(Guzmán-Guillén et al. 2015)	CYN	Neurotoxicity	In vivo, O. niloticus (Nile tilapia)	Activity of acetyle cholinesterase (AChE), physiological changes and degradation of neurons	42.4 µg/L in water	LOEC: Effect observed*
(Falfushynska et al. 2021)	CYN	Oxidative stress	In vivo, zebrafish liver	Levels of thiobarbituric acid reactive substances and protein carbonyls	20 µg/L in water	LOEC: 20 µg/L*
(Falfushynska et al. 2021)	CYN	Genotoxicity	In vivo, zebrafish liver	mRNA expression levels of genes involved in DNA damage repair	20 µg/L in water	LOEC: 20 µg/L*
(Falfushynska et al. 2021)	CYN	Neurotoxicity	In vivo, zebrafish liver	Acetyl cholinesterase activity AChE, in brain	20 µg/L in water	LOEC: 20 µg/L*
(Hercog et al. 2020a)	CYN	Genotoxicity	In vitro, HepG2 spheroids derived from human liver cells	Changes in the transcription of genes	0.125, 0.25, and 0.5 µg/mL.	LOEC: 0,5 µg/mL*

				involved in DNA damage response		
(Hercog et al. 2020b)	CYN	Genotoxicity	In vitro, HepG2 human liver cells	γH2X formation which indicates DNA double strand breaks (DSBs)	0.5 µg/mL	LOEC: 0,5 µg/mL*
(Raška et al. 2019)	CYN	Oxidative stress	In vitro, HL1-hT1 Human liver stem cells	Amount of ROS	415.4 µg/L	No observed effect
(Raška et al. 2019)	CYN	Genotoxicity	In vitro, HL1-hT1 Human liver stem cells	Amount of DNA double-strand breaks (DSBs)	415.4 µg/L	No observed effect
(Puerto et al. 2018)	CYN	Genotoxicity	In vitro, mouse lymphoma cell line L5178YTk	Frequency of micronuclei in binucleated cells	0-1,35 µg/mL In the absence of S9 fraction 0-2 µg/mL in the presence of S9 fraction	LOEC: Absence of S9 fraction No observed effect Presence of S9 fraction 0.25 µg/mL NOEC: Presence of S9 fraction 0.13 µg/mL

(Puerto et al. 2018)	CYN	Genotoxicity	In vitro, caco-2-cells, derived from human colon carcinoma.	Amount of general DNA damage	0.625, 1.25, and 2.5 µg/mL	No observed effect
(Humpage et al. 2000)	CYN	Genotoxicity	In vitro, human WIL2-NS lymphoblastoid cells	Amount of micronuclei (MN) formation	1, 3, 6, and 10 µg/mL. 24 and 48 hours	LOEC: After 24 h 6 µg/mL NOEC: After 24 h 3 µg/mL
(Gutiérrez-Praena et al. 2011)	CYN	Oxidative stress	In vitro, PLHC-1 cell line (fish)	GSH levels	2, 4, and 8 µg/mL	LOEC: 2 µg/mL*
(Gutiérrez-Praena et al. 2011)	CYN	Oxidative stress	In vitro, PLHC-1 cell line (fish)	Reactive oxygen species (ROS) content	2, 4, and 8 µg/mL	LOEC: 2 µg/mL*
(Gutiérrez-Praena et al. 2011)	CYN	Oxidative stress	In vitro, PLHC-1 cell line (fish)	γ-glutamylcysteine synthetase (GCS) activity	2, 4, and 8 µg/mL	LOEC: 2 µg/mL*
(Đorđević et al. 2017)	CYN	Genotoxicity	In vivo, male albino Wistar rats	DNA damage	79.80 µg CYN per kg body weight which was between 220–250 g.	LOEC: Effect observed*

(Đorđević et al. 2017)	CYN	Oxidative stress	In vivo, male albino Wistar rats	Level of antioxidant parameters such as glutathione (GSH),	79.80 µg CYN per kg body weight which was between 220–250 g. 24 h	LOEC: Effect observed*
(Đorđević et al. 2017)	CYN	Oxidative stress	In vivo, male albino Wistar rats	Level of antioxidant parameters such as superoxide dismutase (SOD)	79.80 µg CYN per kg body weight which was between 220–250 g. 24 and 72 h	LOEC: Effect observed*
(Đorđević et al. 2017)	CYN	Oxidative stress	In vivo, male albino Wistar rats	Level of antioxidant parameters such as catalase (CAT)	79.80 µg CYN per kg body weight which was between 220–250 g. 24 and 72 h	LOEC: Effect observed*
(Đorđević et al. 2017)	CYN	Oxidative stress	In vivo, male albino Wistar rats	Level of antioxidant parameters such as thiobarbituric acid-reactive substances (TBARS)	79.80 µg CYN per kg body weight which was between 220–250 g. 24 and 72 h	LOEC: Effect observed*

(Sieroslawska & Rymuszka 2015)	CYN	Oxidative stress	In vitro, CLC cell leucocyte line of carp (Cyprinus carpio L.)	The production of reactive oxygen species (ROS)	0.1, 0.5 and 1.0 µg/mL	LOEC: 0.1 µg mL*
(Sieroslawska & Rymuszka 2015)	CYN	Oxidative stress	In vitro, CLC cell leucocyte line of carp (Cyprinus carpio L.)	The total superoxide dismutase (SOD) activity	0.1, 0.5 and 1.0 µg/mL	LOEC: 0.1 µg mL* No significant result in higher concentrations**
(Sieroslawska & Rymuszka 2015)	CYN	Oxidative stress	In vitro, CLC cell leucocyte line of carp (Cyprinus carpio L.)	The total glutathione (GSH + GSSG) levels and the ratio of reduced (GSH) to oxidized (GSSG) glutathione (GSH/GSSG)	0.1, 0.5 and 1 µg mL	LOEC: 0.1 µg/mL*
(Sieroslawska & Rymuszka 2015)	CYN	Genotoxicity	In vitro, CLC cell leucocyte line of carp (Cyprinus carpio L.)	Formation of micronuclei	0.1, 0.5 and 1 µg/mL	LOEC: 1 µg/mL NOEC: 0.5 µg/mL

(Sieroslawska & Rymuszka 2015)	CYN	Genotoxicity and oxidative stress	In vitro, CLC cell leucocyte line of carp (Cyprinus carpio L.)	The levels of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OHdG)	0.1, 0.5 and 1 µg/mL	LOEC: 0.5 µg/mL* No significant result in higher concentrations**
(Štraser et al. 2013)	CYN	Oxidative stress	In vitro, human liver HepG2 cells	The formation of intracellular Reactive Oxygen Species (ROS)	0.05, 0.1, and 0.5 µg mL	LOEC: 0.05 µg/mL*
(Štraser et al. 2013)	CYN	Oxidative stress and genotoxicity	In vitro, human liver HepG2 cells	The level of oxidative DNA damage	0.125, 0.25, and 0.5 µg mL	LOEC: 0.25 µg/mL NOEC: 0.125 µg/mL
(Gutiérrez-Praena et al. 2012a)	CYN	Oxidative stress	In vitro, Caco-2 cell line, derived from human colon carcinoma	Reactive oxidative species (ROS) content	0.625, 1.25, and 2.5 µg/mL	LOEC: 1.25 µg/mL NOEC: 0,625 µg/mL

						No significant result in higher concentrations**
(Gutiérrez-Praena et al. 2012a)	CYN	Oxidative stress	In vitro, Caco-2 cell line, from human colon carcinoma	Glutathione (GSH) levels	0.625, 1.25, and 2.5 µg/mL	LOEC: 2.5 µg/mL NOEC: 1.25 µg/mL
(Gutiérrez-Praena et al. 2012a)	CYN	Oxidative stress	In vitro, Caco-2 cell line, from human colon carcinoma	Activity of γ-glutamylcysteine synthetase (GCS)	0.625, 1.25, and 2.5 µg/mL	LOEC: 2.5 µg/mL NOEC: 1.25 µg/mL
(Žegura et al. 2011)	CYN	Genotoxicity	In vitro, human peripheral blood lymphocytes (HPBLs)	DNA damage	0.05, 0.1, and 0.5 µg/mL 4 and 24 h	LOEC: After 4 hours 0.5 µg/mL After 24 h 0,05 µg/mL* NOEC: After 4 hours 0.1 µg/mL

						After 24h no NOEC
(Žegura et al. 2011)	CYN	Genotoxicity	In vitro, human peripheral blood lymphocytes (HPBLs)	Formation of micronuclei cells (MNi cells)	0.05, 0.1, and 0.5 µg/mL 4 and 24 h	LOEC: After 4 h 0.5 µg/mL After 24 h 0,1 µg/mL NOEC: After 4 hours 0,1 µg/mL After 24 h 0,05 µg/mL
(Žegura et al. 2011)	CYN	Genotoxicity	In vitro, human peripheral blood lymphocytes (HPBLs)	Formation of micronuclei (MNi)	0.05, 0.1, and 0.5 µg/mL 4 and 24 h	LOEC: After 4 h 0,1 µg/mL After 24 h 0,05 µg/mL* NOEC:

						After 4 h 0,05 µg/mL After 24 h no NOEC
(Žegura et al. 2011)	CYN	Genotoxicity	In vitro, human peripheral blood lymphocytes (HPBLs)	Amount of nuclear buds (NBUDs) in binucleated cells.	0.05, 0.1, and 0.5 µg/mL 4 and 24 h	LOEC: After 4 h 0,05 µg/mL* No significant result in higher concentrations** After 24 h 0,1 µg/mL No significant result in higher concentrations** NOEC: After 24 h 0,05 µg/mL

(Žegura et al. 2011)	CYN	Genotoxicity	In vitro, human peripheral blood lymphocytes (HPBLs)	Amount of nucleoplasmic bridges (NPB)	0.05, 0.1, and 0.5 µg/mL	No observed effect
(Humpage et al. 2005)	CYN	Oxidative stress	In vitro, hepatocytes from the livers of adult male albino Swiss mice	The levels of glutathione (GSH)	0.02077 µg/mL to 10.385 µg/mL	LOEC: 0.4154 µg/mL NOEC: 0.2077 µg/mL
(Humpage et al. 2005)	CYN	Oxidative stress	In vitro, hepatocytes from the livers of adult male albino Swiss mice	Malondialdehyde (MDA) levels	0.02077 to 10.385 µg/mL	LOEC: No effect observed
(Humpage et al. 2005)	CYN	Genotoxicity	In vitro, hepatocytes from the livers of adult male albino Swiss mice	Level of DNA damage	0.02077 to 0.2077 µg/mL	LOEC: 0.02077 µg/mL*

*In experiments where the lowest concentration tested yielded measurable results and no determined NOEC, the Lowest Observed Effect Concentration (LOEC) is possible to be lower than the tested concentrations. Consequently, the reliability of the results is diminished.

**In studies where concentrations higher than LOEC did not yield a significant effect, there is no logical dose-response relationship. The reliability of the results is therefore questioned.

3.2 Which Methods Have Been Employed to Investigate the Toxicity of These Substances?

Between in vitro and in vivo testing a variety of methods were employed. The primary factor setting them apart is the interaction of the toxin, with in vitro experiments focusing on individual cells and in vivo experiments encompassing interactions within an organism. When it comes to the exposure of the toxin, in vitro experiments involved exposing the cells by implementing the toxin in a controlled solution media, while in vivo tests had more options. The toxin was either injected directly into the fish (Osswald et al. 2013) or rat (Đorđević et al. 2017), added to the water (Falfushynska et al. 2021), or implemented into the feed (Gutiérrez-Praena et al. 2012b). Mitrovic et al. (2004) also performed in vivo experiments on aquatic plants where the toxin was mixed in the water.

Despite the difference between in vitro and in vivo tests, the variables measured to investigate oxidative stress in the two model systems, were found to be similar. According to Table 1, one regularly implemented method to measure oxidative stress was to detect the change in activity of enzymes that are involved in the defensive response. For example, peroxidase (POD), glutathione S-transferase (GST), catalase (CAT), superoxide dismutase (SOD), γ-glutamylcysteine synthetase (GCS), glucose-6-phosphate dehydrogenase, soluble or cytosolic glutathione S-transferases (cGST), glutathione peroxidase (GPx) and glutathione disulfide (GSSG). The measurement of the antioxidant glutathione (GSH) is also shown in the table to be a common biomarker and is involved in the defensive mechanism.

Other common indicators, which are not directly involved in the defensive response or enzymatic processes, but rather serves as an outcome or product of oxidative stress, have also been measured. For example: reactive oxygen and nitrogen species (ROS and RONS), malondialdehyde (MDA), lipid peroxidation (LPO), protein carbonyl content, thiobarbituric acid (TBA), glutathione disulfide (GSSG) and 8-OHdG. Sieroslawska and Rymuszka (2015) who measured 8-OHdG, combined their oxidative stress study with a genotoxicity study. They measured 8-OHdG through using fluorometric OxyDNA assay, and as 8-OHdG is a biomarker for oxidative stress it is an oxidation product of DNA, indicating that DNA damage, a genotoxic effect, was induced as well.

The remaining methods for measuring genotoxicity differed entirely from those investigating oxidative stress. The most common technique was the standard and enzyme-modified comet assay, which evaluates DNA damage (Žegura et al. 2011; (Puerto et al. 2018). The comet assay was utilized both in vivo and in vitro studies. Another prevalent method, which was only used in vitro, was the measurement of micronuclei through a cytokinesis-block micronucleus assay (CBMN), which

detects chromosomal abnormalities (Sieroslawska and Rymuszka 2015). In combination with measuring micronuclei, nuclear buds (NBUDs) and nucleoplasmic bridges (NPB), were also quantified and counted as indicators for genotoxicity, also by using CBMN-assay. The third and last method used to detect genotoxicity was flow cytometry which measured the phosphorylated form of histones, γ-gamma-H2AX, an indicator of DNA damage (Zhong et al. 2020).

In comparison to methods used to assess oxidative stress and genotoxicity, where enzymes or DNA fragments were measured, methods to detect neurotoxicity were simply to observe behavioral changes, such as abnormal swimming in fish. Behavioral changes were only used as a method in vivo. Additionally, techniques such as measuring the activity of acetylcholinesterase, both in the brain and in muscles, were employed. Furthermore, physiological changes were observed alongside histopathological analysis of brain tissue from model organisms, revealing degradation or changes of the composition of neurons. The investigation of the viability of nerve cells was also a utilized method where Takser et al. (2016) used a so-called MTT assay.

The techniques for neurotoxicity range from behavioral observations to biochemical assays. However, they can also be combined with detecting genotoxicity. Rabelo et al. (2021) examined neurotoxicity by the detection of DNA damage in fish brain using, the previously mentioned, comet assay. The methods detecting neurotoxicity resulted in more diverse findings, such as behavior pattern, neurotransmitters, the viability of individual cells and composition of brain tissue, in comparison to oxidative stress and genotoxicity.

3.3 For the respective toxins: which parameter of neuro- genotoxicity or oxidative stress, seems to be most or least sensitive?

Both anatoxin and cylindrospermopsin induced all three toxicities. An analysis of the findings related to anatoxin, reveals that there were more studies made on oxidative stress and neurotoxicity compared to the research on genotoxicity. Among the toxicities observed in in vitro studies, genotoxicity had the highest LOEC, whereas oxidative stress demonstrated the lowest LOEC. These preliminary findings from the in vitro tests, suggest that oxidative stress is the more sensitive toxicity parameter compared to neuro- and genotoxicity. It also indicates that genotoxicity seems to be the least responsive screening system when investigating ATX in vitro.

Upon examining anatoxin and the results from the in vivo research, no studies were found about genotoxicity. However, the comparison of the ATX's potency in

inducing oxidative stress and neurotoxicity in vivo appears to be difficult. Experiments where the toxin was mixed in the water, and using μg/g, the combined results from the neurotoxicity studies had the lowest LOEC compared to the results from the oxidative stress studies. This indicates that the neurotoxicity investigations are a more sensitive screening system.

In contrary, experiments where ATX was injected directly into the organisms, oxidative stress had the lowest LOEC and therefore seemed to be the more sensitive parameter. Interestingly, the results from the neurotoxicity tests, where the toxin was injected, showed great variations depending on which variable was measured. One study by Colas et al. (2020) measured behavioral changes, and the effect was observed at a concentration of 20 μg/g. Conversely, another study conducted by Osswald et al. (2013) measured AChE activity, and observed a significant effect at 0.20 μg/g. The difference in results between the neurotoxicity experiments in vivo, despite investigating the same toxicity, suggests variations in sensitivity or response between different parameters.

Regardless of the difference in LOEC of the recently discussed neurotoxicity experiments, if a comparison would be made between the average value for the two neurotoxic LOEC and the LOEC from oxidative stress, oxidative stress seems to be the more sensitive parameter. The combined result or the average LOEC from the two neurotoxicity tests (LOEC of 20 μg/g and 0.20 μg/g) would be 10.1 μg/g, while the variables measured in oxidative stress showed a LOEC at 0.31 μg/g, were the cellular protein, GST, was studied (Osswald et al. 2013). On the other hand, if the LOEC from the neurotoxicity studies were compared respectively to the result from the oxidative stress research, there would be two outcomes. Between the neurotoxicity test, which observed behavioral changes at 20 μg/g, and the oxidative stress study, oxidative stress is the more sensitive parameter. However, the neurotoxicity test investigating the level of AChE (0.20 μg/g), compared to the result from oxidative stress, is in this case the more responsive parameter. This implies that the variability in LOEC within neurotoxicity studies differ depending on the specific parameter being observed, such as behavioral changes or AChE activity. Thus makes an analysis challenging when discussing whether anatoxin is more likely to cause oxidative stress than neurotoxic effects.

Up until this point, the results from anatoxin from in vitro studies together with the in vivo studies, where the toxin was injected into the organism, has been discussed. Regarding the studies examining the effects of ATX in vivo and when the toxin was added to the water, using μg/mL, oxidative stress seemed to be the more sensitive screening system, in comparison to neurotoxicity. Nevertheless, the concentrations for analyzing neurotoxicity from behavioral changes used concentrations between 0.132 – 0.524 μg/mL (Osswald et al. 2011), while in oxidative stress the concentrations ranged from 5 - 25 μg/mL (Mitrovic et al. 2004). The biggest difference here is that Osswald et al. (2011) investigated behavioral

changes in fish, while Mitrovic et al. (2004) studied cellular proteins in plants. Unfortunately, there is no more data that could provide with a fairer comparison between the neurotoxicity and oxidative stress parameters. Consequently, according to the table, the more sensitive parameter for studying ATX in this case, is by measuring neurotoxicity from behavioral changes in fish, than oxidative stress and cellular proteins in plants.

Regarding cylindrospermopsin, the most extensively studied parameters in vitro and in vivo are oxidative stress and genotoxicity. Focusing on the in vitro studies, the least sensitive parameter in yielding a response is neurotoxicity, while genotoxicity appeared to be the most sensitive, with having the lowest LOEC. In fact, genotoxicity showed significant effect in the lowest concentrations in vivo as well, compared to the other two toxicities. It can therefore be considered the most sensitive screening system out of the three studied parameters, both in vitro and in vivo.

Whether the most sensitive parameter from the findings of in vivo experiments is neurotoxicity or oxidative stress, the only comparison possible is when the toxin is mixed in water and not injected. This is due to the restricted data concerning neurotoxicity. Intriguingly, in this context, oxidative stress and neurotoxicity showed equal LOEC. The reason for the aligned LOEC's is that the results from CYN and in vivo experiments that mixed the toxin into the water, are a total of two separate studies. They used the same variables, measuring the level of acetylcholinesterase as an indicator for neurotoxicity and biomarkers of lipid oxidation to detect oxidative stress. The two studies also used fish as their model organism. In one of the studies, conducted by Guzmán-Guillén et al. (2015), oxidative stress and neurotoxicity were noted of giving effect at the same concentration of 42.4 µg/L. Similarly, another study made by Falfushynska et al. (2021) saw effect at a concentration of 20 µg/L, also observing the same LOEC values for both parameters. Consequently, oxidative stress and neurotoxicity appears to be comparably sensitive parameters.

3.4 For the respective type of toxicity: which substance appears to be the most or least potent?

As the sensitivity of the parameters within the substances ATX and CYN have been evaluated, the investigation continues to perhaps a more intriguing question: which of the two, anatoxin or cylindrospermopsin, appears to be more potent?

Regarding oxidative stress, the more potent substance varies depending on the results from the in vivo tests and the in vitro tests. In in vivo tests, cylindrospermopsin seems to be the most potent, both in the experiments where the

toxin was mixed in the water or food and when it was injected. Nevertheless, anatoxin appears to be more potent in the in vitro tests, however the difference between the results from CYN and ATX in vitro are almost marginable.

Moving on to the toxicity parameter of genotoxicity, the more potent substance appeared to be cylindrospermopsin. The results in this context are comparatively clearer, as ATX resulted in notably higher LOEC values compared to CYN. Simultaneously, there were significantly fewer results regarding ATX and genotoxicity, with no available in vivo studies. A total of two in vitro studies are present in Table 1 and a genotoxic effect was only observed in one of them. The experiment where ATX showed genotoxic effect was conducted by Lakshmana Rao et al. (2002), using rat cells, a response was seen at the concentration of 4 µg/mL, with a NOEC of 2 µg/mL. The highest LOEC value observed from CYN were at 6 µg/mL (Humpage et al. 2000), but most of the values, where significant effect was induced, were around 0.5 µg/mL. Hence, according to the data available in Table 1, the more potent substance is cylindrospermopsin for genotoxicity.

The third and last parameter to analyze is neurotoxicity and yet again, cylindrospermopsin showed higher potency in vivo. Nonetheless, the variables measured exhibit notable disparities, making direct comparisons of the results challenging. The studies regarding CYN examined the level of AChE and DNA damage in fish brain (Falfushynska et al., 2021; Rabelo et al., 2021), while the study on ATX examined behavioral changes in fish (Osswald et al. 2011). The experiment investigating DNA degradation and CYN, conducted by Rabelo et al. (2021) resulted in a LOEC value of 1.0 µg/L and a NOEC value of 0.5 µg/L. In comparison, the behavioral study by Osswald et al. (2011) utilized a higher interval of concentration of ATX, yielding in a higher LOEC. On the other hand, the study by Osswald et al. observed effect of neurotoxicity in the lowest concentration tested – meaning that effect could be induced at even lower concentrations than the ones tested. Consequently, the data regarding ATX and neurotoxicity from in vivo experiments are not enough to state that ATX, in this context, is less potent than CYN. Hence, by looking at the results at first glance, cylindrospermopsin appears to be more potent. However, with a comprehensive examination of the experiments conducted, considering the variables measured and number of studies etc., reaching a conclusion is more complex.

Transitioning to the reports on neurotoxicity studied in vitro, anatoxin seems to be the more potent substance. The results here are easier to analyze. One study examined the viability and components that induced cell death in neurons, in both toxins (Takser et al. (2016). Here, the setup of the experiment was the same for ATX and CYN, whereas ATX yielded the lowest LOEC. In this study, values for NOEC were also attained for both substances, increasing the reliability of the results.

3.5 How Reliable and Sensitive Are These Investigative Methods, and What Challenges Do They Encounter?

The reliability and sensitivity of the investigative methods discussed in the text vary depending on the specific context and the parameters being measured. Many of the techniques used for assessing oxidative stress and genotoxicity demonstrate a high degree of reliability. Firstly, the measured parameters in oxidative stress such as ROS and GST etc., are well-known indicators of oxidative stress levels. Likewise, in the genotoxicity experiments, measuring DNA damage in the form of DNA fragments, micronuclei and the activity of genes involved in DNA repair, are reliable methods for assessing genotoxicity because it provides direct insights into the impact of a substance on the integrity of genetic material.

Secondly, the techniques, e.g. CBMN and comet assay, have been widely used and validated by the scientific community over time, establishing confidence in the reliability and validity of the data obtained from their experiments. However, results where studies did not observe a NOEC value, is less conclusive and reliable, as the true LOEC could be at a lower concentration than tested. Therefore, adjusting the concentrations in the experimental methods would be appropriate to assess accurate toxicity thresholds.

The same principles apply to studies examining neurotoxicity and the measurement of AChE. The measurement of AChE activity serves as reliable indicators of neurotoxic effects, and the techniques employed have been extensively used. On the other hand, behavioral changes as a measurement of neurotoxicity can be more challenging to attribute solely to the toxin the fish has been exposed to. This is due to the present of more uncontrolled parameters, such as genetic differences among individuals, which already can lead to variations in behavior. However, in a properly conducted laboratory experiment, where the only factor distinguishing the exposed animals from the unexposed, is the test substance, the results are considered reliable. Moreover, when conducting toxicity studies, ensuring quality assurance is essential throughout the experimental process.

For all three toxicities, a positive and a negative control were present when conducting the experiments, giving a reference point for assessing credible results. The positive control allows researchers to compare the response of the test substance to a known toxic substance, ensuring the experimental conditions are suitable and the test system is capable of detecting toxic effects. Conversely, the negative control establishes a baseline measurement, helping researchers determine whether any observed effects stem from the test substance itself or normal

variability in the experimental system. This dual approach enhances the accuracy of the study and facilitates more validated interpretation of the data regarding the toxicity of the test substance.

One challenge encountered by all the methods discussed is that, while molecular assessments provide valuable insights into cellular responses, they do not fully capture the broader physiological impact of toxin exposure in humans. Despite the in vitro studies on human leukocytes being good screening tools on the molecular level, there is no possibility to predict how a toxin will behave in a complete human body. The behavioral investigation in fish does not directly translate to the response of a human due to the fundamental differences in anatomy, physiology, and behavior. Yet, collectively, by combining in vivo studies that evaluate systemic and behavioral effects in living organisms, with molecular assessments that identify molecular targets and pathways, offers a more comprehensive understanding of how ATX and CYN could impact human health.

4. Discussion

4.1 What Treatment Methods Exist for Removing or Reducing the Concentration of Cylindrospermopsin and Anatoxin in Drinking Water?

In a study conducted by Lebad et al. (2024) various membranes, NF-270, TRISEP, HYDRACoRe-10, and HYDRACoRe-50, were tested to see how well they could remove ATX and CYN. The first one mentioned being nanofiltration membranes and the latter three being sulfonated polyethersulfone membranes. At first, the membranes NF-270 and HYDRACoRe-50, effectively removed ATX. But over time, their ability to remove it decreased. For CYN, all membranes showed similar initial removal rates, but this also decreased as time went on. These results show that removing cyanotoxins with membranes can be successful; however it is complicated and can change over time, which is important to consider when treating water (Lebad et al. 2024).

Moreover, an investigation into cyanobacteria presence in a French drinking water reservoir during an algal bloom, involved analyzing the treatment plant's purification methods under typical operational conditions. A treatment showing positive results included pre-ozonation and the use of powdered activated carbon (PAC). A combined treatment of established concentrations of pre-ozonation and PAC resulted in complete destruction and removal of the toxins at (Maatouk et al. 2002). Nevertheless, while powdered activated carbon (PAC) can effectively reduce both cyanotoxin levels when used at an optimal dosage, if water treatment facilities face persistent problems with these toxins, they need to use granular activated carbon (GAC) instead (He et al. 2016). In Germany, some waterworks that rely on eutrophic surface water utilize GAC as an efficient technique for the removal of the toxins (Marsálek et al. 2005b). Yet, the effectiveness of GAC can be quite costly and can therefore not be used as a method used in traditional drinking water treatment. Attempts have been made to investigate modified versions of GAC, so-called GAC cap, for the removal of substances such as organic compounds, with success. Yet, their efficacy in removing cyanotoxins remains uncertain (Crowe et al. 2022). Furthermore, bank filtration has been proven to

remove cyanotoxins. In an investigation conducted by Walkenhorst et al. (2021), sand was used as the filtration medium, and the study investigated how effectively this sand filtration setup could remove cyanotoxins under different environmental conditions, such as varying levels of nutrients and cyanotoxins. The different levels of nutrients and cyanotoxins had no impact on the overall ability to remove cyanotoxins, however when the sand was biologically active, it played a crucial role in its reduction. The results suggested that that the removal of cyanotoxins was primarily due to biodegradation facilitated by biologically active sand, rather than sorption, highlighting its potential as a cost-effective and sustainable treatment option (Walkenhorst et al. 2021).

4.2 What Are the Current Knowledge Gaps and Future Research Needs Regarding Cylindrospermopsin and Anatoxin in Drinking Water?

The current understanding of cylindrospermopsin and anatoxin in drinking water highlights several areas where further research is needed. As techniques for assessing their effects have been developed, there's a lack of standardized testing methods, particularly considering the differences between in vitro and in vivo approaches. During comparisons of the potency of cylindrospermopsin and anatoxin it revealed complexities. For example, in vivo tests suggest that cylindrospermopsin may induce oxidative stress more effectively, while anatoxin exhibits higher potency in vitro. While both in vitro and in vivo studies offer valuable insights into cyanotoxin effects, their disparate methodologies often hinder direct comparison and integration of results. Future research endeavors might concentrate on establishing standardized protocols or computational models that facilitate the harmonization of data obtained from these distinct approaches. By doing so, researchers can achieve a more cohesive understanding of the physiological ramifications of cyanotoxin exposure across various biological systems.

Additionally, when discussing which parameter is more sensitive, neurotoxicity or oxidative stress, the comparison is difficult due to the variables discussed being changes in behavior and shifts in glutathione levels. As the measured variables differ remarkably, and one set of experiments employing concentrations as high as 20 µg/L and the other as low as 0.1 µg/L, the disparity complicates direct comparison. This emphasizes the necessity for standardized methodologies to facilitate accurate comparisons.

A more specific research gap is the research regarding anatoxin and genotoxicity. Especially in vivo since no reports were found regarding that specific

toxicity parameter and model system. In general, much more research was found about CYN. Due to the increasing concentration of both cyanotoxins in freshwater lakes around our planet, unraveling the toxicological profile of anatoxin is much relevant.

The urgency to enhance standardization and address research gaps in toxicity studies stems from the escalating apprehension regarding the prevalence of cyanobacteria in our drinking water. Unfortunately, these toxins present challenges for water treatment due to their resistance to conventional purification methods. While some techniques have been utilized with success for removal of cyanotoxins, the issue regarding the extensive cost remains. Efforts to develop cost-effective techniques, such as GAC cap or bank filtration, for the removal of cyanobacteria from water sources are crucial, particularly if these methods can be integrated into existing water treatment facilities, and especially in freshwater lakes vulnerable to algal blooms.

Addressing these knowledge gaps and conducting further research will contribute to improving water quality and ensuring the safety of drinking water in the presence of cylindrospermopsin and anatoxin. Even so, while filling future research gaps and confronting the knowledge deficiencies is crucial for mitigating the impacts of cyanobacteria-induced algal blooms, it's imperative to acknowledge that these blooms will persist and worsen due to underlying factors such as global warming, urbanization, and sewage discharges. To get into combat with the actual root of the discussed issue, presents an enormous challenge. As a conclusion, in the absence of comprehensive solutions to these broader issues, addressing the identified research needs and knowledge gaps will remain our best course of action.

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