# Effects of cyanobacterial toxins on the human gastrointestinal tract and the mucosal innate immune system

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**Abstract:** Cyanobacterial blooms occur with increasing frequency in freshwater ecosystems, posing a hazard to human and environmental health. Exposure of human to cyanobacterial metabolites occurs mostly via accidental ingestion through contaminated drinking water or during recreational activities and, most frequently, results in gastrointestinal symptoms. Despite the clinical manifestation, cyanobacterial metabolites are rather investigated for their toxicity towards specific organs or tissues, especially hepato-, nephro- and neurotoxicity, then for effects on the gastrointestinal tract and the associated lymphoid tissue.

The aim of this review was to systematically summarize available literature on the effects on the gastrointestinal tract and the mucosal innate immune system and compile the data from both, *in vitro* and *in vivo* studies, focusing on human-health relevant models. Our systematic literature review revealed significant data gaps in the understanding on metabolites breaching the gastrointestinal barrier and the role of the immune system in the establishment of clinical symptoms. Microcystins and cylindrospermopsin were linked to gastrointestinal symptoms, immune system effects or both. Furthermore, implications for cyanobacterial bloom

lipopolysaccharides in gastrointestinal inflammation were reported in several cases, while other metabolites received only minor attention.

The collected data indicate the need for a reassessment of potential enterotoxicity of microcystins and cylindrospermopsin. Additionally, the carcinogenic potential of cyanotoxins, especially microcystins, has to be clarified, as an increasing amount of epidemiological studies show correlations between cyanobacterial blooms and gastrointestinal cancer incidence. Furthermore, other, often highly abundant bioactive metabolites like aeruginosins, have to be toxicologically evaluated at levels also accounting for (sub-)chronic exposure to low concentrations and in combination with naturally co-occurring metabolites, as can be expected in drinking water supplies.

**Keywords:** Cyanotoxin, cyanobacterial bloom, cylindrospermopsin, microcystin, inflammation, diarrhea, gastrointestinal illness, lipopolysaccharide, innate immune system

## Background

Climate change is transforming ecosystems and their composition all over the planet. Among others, temperate climates in the northern hemisphere are experiencing longer and more intense heat periods in summer and increasing $CO_2$-levels are saturating surface waters with dissolved carbonate. In combination with other anthropogenic factors, such as eutrophication, this has been linked to an increase in frequency and intensity of hazardous cyanobacterial blooms in surface waters [1–4].

Cyanobacteria, also known as blue-green algae, are photosynthetic prokaryotes of ancient origin. They are able to produce a large variety of toxic secondary metabolites, cyanotoxins, heterogeneous in structure, activity and stability (reviewed in Buratti et al. [5]). These can be released to water either directly or upon cell lysis with major implications for the directly affected aquatic species, as well as for human and livestock health. For humans, Figure 1 illustrates the organs that are traditionally reported as targets of toxicity, as well as other organs/organ systems affected by cyanobacterial toxins.

As reviewed in Wood [6] and Stewart [7], fatal poisonings of livestock and pets (e.g. dogs) have been reportedly linked to cyanobacterial blooms for decades.

Unlike animals, humans have greater capabilities to access uncontaminated drinking water (underground water or treated surface water) and tend to avoid odorous waters for drinking and recreational activities. Therefore, documented fatal human cases of acute cyanobacteria poisonings have been limited to specific exposure routes, such as hemodialysis treatment with cyanotoxin-contaminated water [8].

Nevertheless, serious adverse health outcomes have been associated also with other, more common exposure scenarios, and linked to both acute and chronic exposures to toxic cyanobacteria and cyanotoxins. During recreational water activities and sports, human beings can be exposed to contaminated water by dermal contact, inhalation, and also ingestion [5]. In the most extreme cases, recreational exposure to cyanobacteria resulted in life-threatening conditions requiring hospitalization, e.g. due to atypical pneumonia [9], pneumonia followed by dyspnea and liver damage [10], or an acute hepatic failure requiring a liver transplant [11]. Gastrointestinal symptoms, including abdominal pain, malaise, nausea, vomiting and diarrhea, were also manifested during these severe poisonings. Interestingly, such gastrointestinal symptoms have been often documented also in the other cases of acute, usually self-limited, adverse health outcomes, which followed recreational contact with cyanobacteria-contaminated water [5,12] (see also sections below). A prospective epidemiological study found an increased incidence of gastrointestinal illnesses in human populations living in the proximity of cyanobacteria-contaminated lakes, even if their recreational contact with the contaminated water was very limited [13,14].

Gastrointestinal irritation and diseases are regularly linked to the occurrence of cyanobacterial blooms, especially to the consumption of cyanobacteria-contaminated drinking water as reported by Levesque et al. [13,14] and reviewed in Svirčev et al. [12]. Acute poisonings in a larger scale have been mostly reported upon consumption of insufficiently treated surface water, often following algicidal treatment of cyanobacteria-infested freshwater reservoirs used as drinking water sources [15,16]. Lysis of cyanobacterial cells upon algicidal treatment releases the intracellular toxic metabolites to the water. While conventional treatment technologies can eliminate intact cyanobacterial cells with toxins quite effectively, these technologies have limited capability for removal of dissolved cyanobacterial toxins, which allows them to reach taps in sufficiently high

concentrations to cause adverse effects in the gastrointestinal system (Table 1). Chronic exposures to drinking water contaminated with cyanobacterial compounds, in some instances in combination with other confounding factors, have been associated with an increased incidence of liver cancers or chronic liver damage and diseases, as reviewed and discussed by Svircev et al [12], and supported by recent findings [17–19]. Interestingly, epidemiologic studies have linked chronic exposures to drinking water contaminated with cyanobacteria and their metabolites also to the increased incidence of colorectal or small intestinal cancer in China, Portugal and Serbia, or stomach cancer mortality in China [12,20,21]. Nevertheless, other studies have not observed such associations between colorectal cancer and exposures to toxic cyanobacteria, or found negative correlation for small intestinal cancer [12,22].

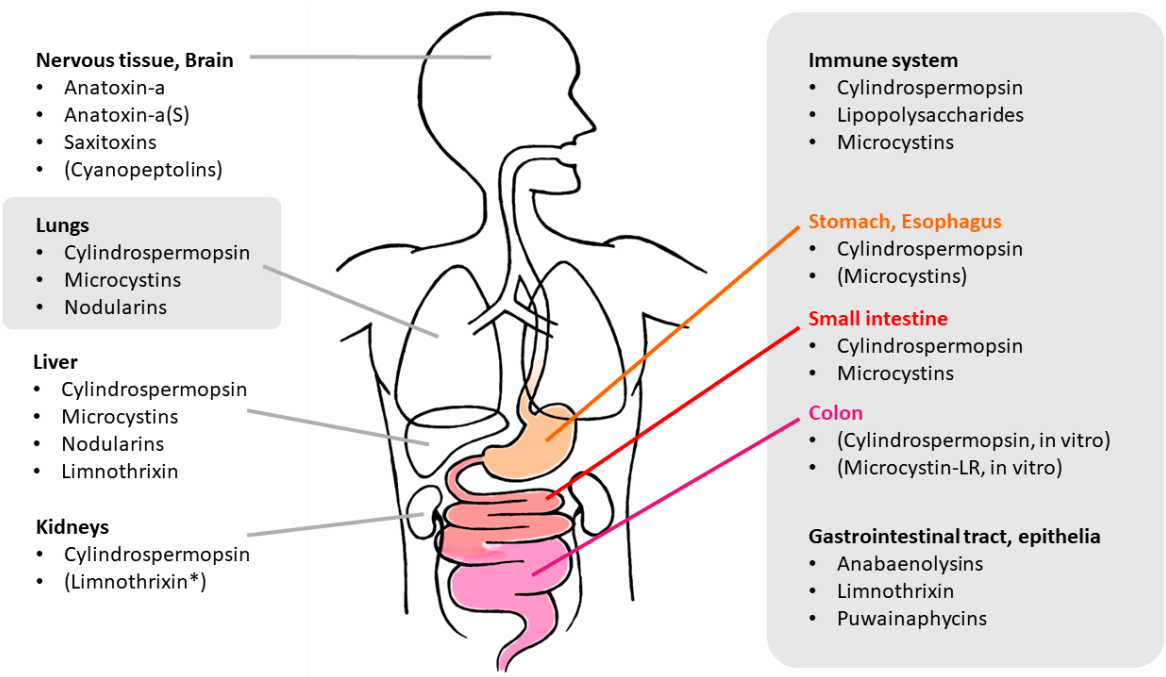


**Figure 1: Organs/organ systems affected by toxic metabolites of cyanobacteria.** Organs traditionally considered targets of toxicity are on the left, organs directly subjected to oral exposure are on the right. Grey background illustrates organs with mucosal surfaces that serve as primary entry portals for environmental and dietary contaminants. The figure depicts inner organs figuratively and not anatomically correct. Data based on literature review, detailed sources are provided in Tables 2-4. *: putative toxin, in parentheses: effect supported by *in vitro* data only (no *in vivo* data available).

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101 **Table 1: Examples of incidents and case studies of acute gastrointestinal illnesses, chronic gastrointestinal illnesses connected to the consumption of cyanobacteria contaminated**
102 **water and chronic gastrointestinal illnesses connected to the recreational activities in cyanobacteria contaminated water.** Ref.: reference(s), n.a.: not assessed, MCs: microcystins,
103 NOD: nodularin, ATX: anatoxin, STX: saxitoxin, LPS: lipopolysaccharide

| Country | Year | Cyanobacteria | Cyanotoxins | Description | Ref. |
|---|---|---|---|---|---|
| **Acute gastrointestinal illnesses connected to the consumption of cyanobacteria contaminated water** | | | | | |
| USA, West Virginia, Charlestone | 1930 | *Microcystis* bloom | n.a. | Massive water blooms of *Microcystis* sp. in the Ohio and Potomac Rivers linked to gastrointestinal illness (abdominal pain, nausea, vomiting, diarrhea) in ≈5000-8000 people out of 60000 inhabitants, despite water treatment by precipitation, filtration and chlorination. | [23] |
| Zimbabwe, Harare | 1960-1965 | *Microcystis* bloom | n.a. | Annual increases of children hospitalizations due to gastroenteritis in a city area supplied with drinking water from a water reservoir during a cyanobacterial bloom decay, no infectious agents detected, populations of children from other areas supplied with water without bloom development unaffected. | [24] |
| USA, Pennsylvania, Sewickley | 1975 | *Schizotrix calciola* | n.a. | Gastrointestinal outbreak in ≈62% of 8000 inhabitants supplied with drinking water from a water source contaminated with cyanobacterium *Schizotrix calciola* | [25,26] |
| Australia, Palm Island | 1979 | *Cylindrospermopsis raciborskii* | CYN implicated | After the use of copper sulfate to control a dense cyanobacterial bloom in water supply, 139 children and 10 adults suffered with severe, hospitalization-requiring, hepatoenteritis and gastroenteritis (malaise, nausea, vomiting, headaches, hepatomegaly, diarrhea, dehydration), with an evidence for liver and renal damage | [15,27]. |
| Brazil, Itaparica Dam | 1988 | *Microcystis* sp., *Anabaena* sp. | n.a. | 2000 gastroenteritis, with 88 cases resulting in death, reported over 42-day period after flooding a newly constructed dam. Cases were restricted to the areas supplied with drinking water from the dam; cyanobacteria were detected in untreated water. No other toxicants or infectious agents were identified. | [28] |
| Sweden, Malmo area | 1994 | *Planktothrix agardhii, Microcystis sp.*, | MCs | Accidental mixing of untreated river water with treated drinking water during a cyanobacterial bloom; 121 people from 304 experienced nausea, abdominal pain, vomiting, diarrhea accompanied with muscle pain, headaches and fever. No pathogens detected upon clinical examinations of patients or in the river water | [29] |
| Australia, South Australia. Murray River | 1995 | Most common: *Dolichospermum circinale, Microcystis aeruginosa, Aphanizomenon* sp., *Planktothrix* sp. | n.a. | Increased incidence of gastrointestinal symptoms in people consuming treated (chlorinated) river water during period of raised cyanobacterial cell counts in the river; gastrointestinal and dermatological symptoms in people using untreated river water for domestic purposes, when compared to people using rain water | [30] |

| | | | | | |
|---|---|---|---|---|---|
| Namibia, Windhoek | 2000 | *Microcystis* sp., *Oscillatoria* sp., *Anabaena* sp., *Merismopedia* sp. | MCs | Liver damage (increased serum levels of liver enzymes) and diarrhea during the occurrence of cyanotoxins and cyanobacteria in treated drinking water, positive correlation between diarrhea and chlorophyll a concentration in the water | [31] |
| Serbia, Uzice | 2013 | *Planktothrix rubescens* | | Cyanobacteria and cyanotoxins found to penetrate into final treated water in December 2013; significantly higher incidence of gastrointestinal, skin and subcutaneous diseases detected during bloom periods in 2012-2015 | [32] |
| USA, Ohio, Lucas County | 2014 | | MCs | Microcystin levels in drinking water reached 3.19 µg/L, a do-not-drink advisory for 3 d was issued. In 16.2% out of >100000 households, at least one person reported physical health symptoms attributed to the advisory; gastrointestinal symptoms were most commonly reported (diarrhea 12.1%, nausea 9.1%, and vomiting 6.2%). Eye and skin irritation, headaches and respiratory symptomes also reported | [33] |
| | **Chronic gastrointestinal illnesses connected to the consumption of cyanobacteria contaminated water** | | | | |
| China, Zhejiang, Haining, | 1977-1996 | n.a. | MCs | Greater incidence of colorectal cancers in patients relying on river and pond water as drinking water source in comparison to people using underground well water or tap water. Microcystins detected in river and pond water, their concentrations correlated with the cancer incidence | [21] |
| China, Jiangsu, Wuxi | | n.a. | MCs | Microcystin in drinking water positively correlated with male overall cancer mortality and male stomach cancer mortality, but negatively correlated with male intestinal cancer mortality | [34]* |
| USA, Florida | 1981-1998 | n.a. | n.a. | No significant associations between the incidence of colorectal cancer in people living within the area supplied from surface water treatment plant and people supplied by underground wells found in geographic information system-based study | [22]* |
| Serbia | 1999-2008 | *Microcystis* sp., *Aphanizomenon* sp., *Anabaena* sp., *Planktothrix* sp. | MCs | Geographical incidence of 13 cancers (brain; bronchus and lung; heart, mediastinum, and pleura; ovary; testis; kidney; stomach; small intestine; colorectum; retroperitoneum and peritoneum; leukaemia; malignant melanoma of skin and primary liver cancer) positively correlated with the occurrence of cyanobacterial blooms and toxins | [20] |
| Portugal | 2000-2010 | *Microcystis aeruginosa, Aphanizomenon* sp., *Oscillatoria* sp. | n.a. | Populations exposed to cyanobacteria-contaminated drinking water had higher serum levels of liver enzymes, and higher incidence of investigated cancers (liver, colon and rectum cancer) | [35]* |
| USA, Ohio, Celina (Mercer County) | 1996-2008 | *Aphanizomenon* sp., *Microcystis* sp., *Anabaena* sp., *Planktothrix* sp. | MC, CYN, ANTX-A, STX | Pperiodically supplied with cyanobacteria-contaminated surface water from Grand Lake St. Marys; comparison of cancer incidence (hepatocellular and colorectal cancer) was inconclusive compared to two groundwater supplied cities | [36]* |
| | **Chronic gastrointestinal illnesses connected to the recreational activities in cyanobacteria contaminated water** | | | | |

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| Canada, Saskatchewan | 1959 | *Microcystis* sp., *Dolichospermum circinale* | n.a. | Ddespite animal deaths and warnings against recreational use, people swam in cyanobacteria-contaminated lake; 13 people suffered from headaches, weakness, nausea, stomach cramps, vomiting, painful diarrhea, muscle and joint pains. No pathogens detected, cyanobacteria found in the vomit and stool of one patient | [37] |
| USA, Pennsylvania | 1979 | *Anabaena* sp. | n.a. | Hay fever-like and gastrointestinal symptoms following recreational water activities in cyanobacteria-contaminated lake, no pathogenic agents detected | [38] |
| Great Britain, Staffordshire | 1989 | *Microcystis* sp. | n.a. | 10 out of 20 army recruits suffered from gastrointestinal symptoms (vomiting, diarrhea, abdominal pain) and other health issues (lips blistering, sore throat) after swimming and canoe training in water with cyanobacterial bloom. Two recruits developed atypical pneumonia. | [9] |
| Australia, New South Wales, Victoria | 1995 | *Microcystis* sp., *M. aeruginosa, Anabaena* sp., *Aphanizomenon* sp., *Nodularia spumigena* | n.a. (hepato-toxicity by bioassay) | Epidemiological study (777 exposed, 75 control) reported positive correlation between exposure to cyanobacteria during recreation and diarrhea, vomiting, flu-like symptoms, skin rashes, mouth ulcers, eye and ear irritations observed 2-7 days after the exposure. Severity of the symptoms depended on bloom density and duration of the activity | [39] |
| Australia, New South Wales, Florida | 1999-2002 | cyanobacteria | n.a.; MCs, CYN, ATX | Respiratory symptoms, gastrointestinal illness, eye and ear irritation associated with recreational use of cyanobacteria and cyanotoxin contaminated water | [40] |
| Great Britain, Littleborough | 1996 | *Planktothrix agardhii* | MCs | Cyanobacterial bloom producing microcystins caused vomiting, fever, facial rashes, asthma, and dry sporadic cough in 11 cadets practicing canoe-capsizing | [41] |
| Finland, lakes Sompanen, Salajarvi, Iso-Kukkanen | 2002-2003 | *Dolichospermum lemmermannii* | STXs | Recreational activities in lakes with cyanobacterial bloom caused skin rashes, eye irritations, fever and abdominal pains in 2-10 year old children | [42] |
| Argentina, Salto Grande Dam | 2007 | *Microcystis* sp. | MCs | 19-year old man practicing jet ski stayed for more than 2 h in dense cyanobacterial bloom, developed gastrointestinal malaise, nausea, vomiting, muscle weakness a few hours later, his condition worsened during next 4 days, developed into pulmonary problems and dyspnea, followed by hepatotoxicosis | [10] |
| Canada | 2009 | n.a. | MCs, LPS | Increased incidence of gastrointestinal symptoms (diarrhea, vomiting, eventually nausea, fever, abdominal cramps) in residents with full recreational contact at cyanobacteria-contaminated lake (swimming, waterskiing, windsurfing etc.). These symptoms reported also in case of limited contact with contaminated water (fishing, watercraft using but not launching). Significantly higher incidence of various other symptoms (muscle pain, skin symptoms, ear symptoms) in populations supplied with treated surface water from cyanobacteria contaminated supplies. The symptoms correlated with concentrations of lipopolysaccharides | [13,14] |

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|---|---|---|---|---|---|
| Finland | 2010 | n.a. | MCs, NOD, ATX, STX, LPS | Brackish and freshwater localities: health issues reported upon recreational activities in cyanobacteria-contaminated water: fever 58%, gastrointestinal symptoms 53%, nausea 34%, skin irritation 34%, headaches 32%, eye-ear-nose-throat irritation 29%, others 16%. While concentrations of cyanotoxins and LPS relatively low, *Aeromonas sp.* virulence genes were detected frequently in the bacteria isolated from the water samples | [43] |
| USA | 2009-2010 | n.a. | MCs, STX, CYN, ATX | Ohio, New York, Washington: 11 outbreaks of cyanobacterial blooms resulted in at least 61 illnesses (two hospitalizations, no known death), effects included dermatologic signs or symptoms (8); gastrointestinal signs or symptoms (8); respiratory signs or symptoms (6); fever (5); headache (4); neurologic signs or symptoms (4); ear symptoms (5); and eye irritation (3). In each of the outbreaks for which oral exposure was reported, affected persons had gastrointestinal signs or symptoms | [44] |
| USA | 2011 | *Microcystis* sp. | MCs | Kansas, Milford Lake: 7 reports of human illnesses confirmed as associated with cyanobacterial blooms, primary symptoms included: 71% eye and upper respiratory tract irritation, 29% rash, 14% gastrointestinal. The primary route of exposure included direct contact | [45] |
| Uruguay | 2015 | *Microcystis* sp. | MCs | Carrasco and Malvín beaches, Montevideo: a family with a 20-month-old child suffered gastrointestinal symptoms after recreational activities, the symptoms were self-limited except in the child, who was hospitalized with diarrhea, vomiting, fatigue, and jaundice. Serum levels of liver enzymes and bilirubin indicated liver damage resulting in acute liver failure 5 days later (liver transplant 20 days later). Microcystins detected in the liver tissue | [11] |

* retrieved from [12]

## The gastrointestinal system and its link to the immune system

Upon oral exposure, which is the most relevant route of exposure to cyanobacterial metabolites [46], gastrointestinal epithelia are the first barrier to be overcome before systemic exposure, hence the gastrointestinal tract is a major entry portal for cyanobacteria and their bioactive metabolites into an organism's system. Additionally, the gastrointestinal system is highly associated with the immune system. Besides harboring commensal microbiota, ingested foodstuffs, pathogens and environmental contaminants are passed through the intestines. These give rise to many toxic or antigenic compounds that may trigger inflammatory responses. To maintain a homeostatic environment and prevent exaggerated immunological reactions when inappropriate, there are several gut-specific adaptations from all compartments involved: the intestinal epithelium, the gut-associated lymphoid tissue and the microbiome.

Interactions between the microbiome and the mucosal immune system or the gastrointestinal tract (GIT) play a critical role in the tolerance of intestinal microbiome and its specific antigens by host-defense systems to avoid permanent inflammatory states [47]. There are indications, that exposure to the cyanobacterial toxin microcystin-LR alters the taxonomic composition of the microbiome, as well as the expression of functional genes in the GIT of mammals (mice, rats) [48,49].

Anatomically, the gastrointestinal passage consists of the esophagus terminating into the stomach that connects via the pylorus to the small intestine (duodenum, jejunum, ileum in descending order). The small intestine connects to the large intestine (caecum, colon, rectum) via the ileocecal valve and terminates with the anus [50]. While the digestively highly active small intestine is characterized by structured epithelia with maximum surface area, the colon is lined by a thick mucus layer enabling effective water reabsorption while preventing tissue invasion of the essential commensal microbiota that reside predominantly in this part of the GIT [50,51]. Besides commensal bacteria residing in the gut, also the intestinal luminal content has immune-modulatory activity itself. The varying concentrations of luminal dietary constituents "along the length of the intestine are likely to have an important impact on regulating regionalized immune cell compartmentalization and functionality." [51].

How do the GIT and the immune system accomplish the task of protective immunity and pathogen clearance while tolerating commensal microbes? In the colon, the mucus secreted by the epithelial goblet cells is antimicrobial and therefore poses a physical barrier for bacteria [50 and references therein]. The mucus production is controlled by soluble immune mediators also known as cytokines. Cytokines can be secreted by epithelial or immune cells and modulate the immune response. For example, tumor growth factor-beta (TGF-β) and retinoic acid promote regulatory T cell (Treg) differentiation in the gut, responsible for the homeostatic equilibrium in the intestines to large extent [51–53]. On the other hand, pro-inflammatory interleukin (IL) IL-1, IL-6 and tumor necrosis factor-alpha (TNF-α), are released by phagocytic cells upon activation of Toll-like receptors (TLRs) sensing pathogen-associated molecular patterns (PAMPs).

Intestinal phagocytic macrophages residing in the gut mucosa are sessile and they constitutively produce high amounts of IL-10 enabling them to limit the inflammation via effective scavenging. It results in low production of inflammatory cytokines or oxidative agents upon phagocytosis or TLR stimulation compared to macrophages in other parts of the body [54, p. 505]. Meanwhile, dendritic cells are mobile and act as major antigen presenting cells migrating to secondary lymphoid organs and priming B and T cells that then can be recruited to the gut-associated lymphoid tissue and eventually secrete the protective immunoglobulin A into the lumen [54].

These integrated mechanisms, spatially restricted to the intestinal proximity, allow the immune system to remain unresponsive to commensal microbiota and dietary constituents in the gut lumen and to launch a robust immune defense reaction when these microorganisms or compounds cross the epithelial barrier and invade tissues and the organism.

## Microcystin effects

Microcystins (MCs), non-ribosomal cyclic heptapeptides, are the most common toxins in cyanobacterial blooms and most extensively studied cyanotoxins (reviewed in Testai et al [55] and Buratti et al. [5]). MCs are synthesized via non-ribosomal polyketide/peptide synthetase multienzyme complexes encoded by the *mcy* gene cluster. The general structure of MCs is a cyclo (-D-alanine[1] -L-X[2] -D-erythro-B-methylaspartic acid[3] -L-Y[4] -Adda[5] -D-glutamate[6] -N-methyldehydroalanine[7]). Adda represents (all-*S*,all-*E*)-3-Amino-9-methoxy-2,6,8-trimethyl-10-

phenyldeca-4,6-dienoic acid, which is an unique feature characteristic for all MCs. Variable L-amino acids can be present in positions 2 and 4, for example leucine (L) and arginine (R), this structural variant is then called microcystin-LR (MC-LR). More than 240 structural variants of MCs have been reported, differing not only in the amino acids at positions 2 and 4, but also other modifications, such as demethylations of N-methyldehydroalanine, methylaspartic acid or Adda [56].

Due to their hydrophilic nature, MCs cannot readily enter the cells of exposed organisms but require an uptake mechanism. Upon swallowing, MCs overcome the gastrointestinal epithelial barrier and enter blood circulation [57–60]. Uptake into hepatocytes is facilitated by organic anion transport polypeptides (OATPs) of the bile acid transport system, particularly OATP1B1, 1B3 and, to a minor extent, OATP1A2 that are highly expressed in parenchymal hepatocytes [61,62].

Of the 12 identified OATPs only few (OATP1B1, 1B3, 2B1 and 1A2) have been extensively studied due to their expression in the tissues recognized as the main target of MC toxicity [63,64]. Of these four OATPs, only OATP2B1 that is not involved in the uptake of MCs seems to be functionally relevant in the intestines, while the expression and function of MC-transporting OATP1A2 in the intestines is still being discussed [62–65]. The expression of OATP1B1 and 1B3 is considered specific to the liver [63], hence their contribution to the intestinal uptake of MCs is probably negligible. Other OATPs functionally expressed in the human intestinal tract are OATP4A1, 3A1 and 2A1, however, these have not been examined for their involvement in MC uptake [62,63,66]. Similarly to the GIT, cells of the innate immune system only express a limited set of OATPs. Transcriptomic analyses revealed high expression of OATP2B1 mRNA and lower levels of OATP3A1, 4A1 and 4C1 while no transcriptomic indication for OATP1A1, 1A5, 1B1, 1B2, 1B3 or 1C1 expression in macrophages was found [67–69]. Furthermore, functional studies on drug transport via OATP2A1 and 2B1 in macrophages indicate their functional expression [70,71]. Moreau et al. [67] also report intermediate to low expression levels of OATP3A1 and 4C1 in monocytes, but their role in MC cellular uptake is unclear.

Once inside cells, MCs inhibit the ubiquitous protein phosphatases 1 and 2A (PP1/2A) due to the interaction of Adda-methyldehydroalalnine moiety with the catalytic site of the enzyme, including a covalent binding between methyldehydroalanine of MCs and cysteinyl residue at the catalytic site

191 of the enzyme [5]. The consequence of protein PP1/2A inhibition by MCs is hyperphosphorylation
192 of cytoskeletal proteins, disruption of intracellular signaling, inhibition of DNA repair, mitochondrial
193 alternation and oxidative stress, DNA damage, apoptosis and necrosis induction [5,72]. Protein
194 phosphatases play a crucial role in the intracellular signaling by dephosphorylating, and thereby
195 deactivating, proteins like Raf, MEK, AKT (PP2A, action as tumor suppressor) [73]. Furthermore, MC-
196 LR has been shown to stimulate pro-inflammatory cytokine production in murine macrophages
197 (RAW 264.7) in a phosphatase-independent manner via activation of Toll-like receptors [5,68], to
198 promote tumor growth and possibly neoplastic transformation and carcinogenesis [74–76].

199 Of >240 congeners, MC-LR is considered to be one of the most common, abundant and toxic
200 structural variant, with a lethal dose killing 50% of the orally exposed mice (LD50, acute oral toxicity)
201 of 5 mg/kg bodyweight and a no-observed adverse effect level ($NOAEL_{liver}$) of 40 µg/kg/day (chronic
202 toxicity, 13 weeks repeated-dose exposure) [2,46,77–81]. Modifications of MC molecule, including
203 modifications of Adda-methyldehydroalanine region as well as other parts, such as variable amino
204 acid residues at positions 2 and 4, can lead to the reduction of PP1/2A inhibition potencies
205 [61,62,82–86]. However, the observed differences in acute *in vivo* toxicity of MCs [86] cannot be
206 exclusively attributed to the differences in PP1/2A inhibition among different MC variants. OATP-
207 mediated cellular uptake of MCs seems to be also structure dependent, which contributes to the
208 differences between cytotoxicity of individual structural variants without a clear relationship to
209 PP1/2A inhibition potency [61]. MCs are detoxified by conjugation with glutathione (GSH) which is
210 catalyzed by the activity of biotransformation enzyme glutathione-*S*-transferase. Reaction is
211 followed by MC-GSH conversion with by gamma-glutamyltranspeptidase and then cysteinylglycine
212 dipeptidase into MC-Cystein conjugate. This biotransformation reduces cellular uptake of the toxin
213 and/or facilitates its excretion that most likely occurs via P-glycoprotein or multidrug resistance
214 proteins, hence alters the ability to inhibit PP1/2A and decreases toxicity [84,87,88].

215 Depending on the MC congener, the dose and the duration of exposure, MCs cause necrotic,
216 apoptotic or cell-proliferative changes. Due to the high abundance of OATP1B1 and 1B3 in
217 parenchymal hepatocytes, the high metabolic activity and detoxifying function, toxic effects often
218 manifest in the liver, even though the first site of action upon water-borne contaminants are

probably mucosal epithelia of the GIT (Table 2). In fact, oral exposure of mice to MC-LR caused erosion of surface epithelial cells of the small intestine and accumulation of MC-LR in the villi (Table 2) [88]. DNA damage in intestine and colon of mice after intraperitoneal exposure was reported [89], as well as increased in apoptotic indices in different parts of the murine intestine [90]. In both, the small and large intestine (caecum), MC-LR was detected in the mucus of goblet cells, indicating the excretion of MCs into the GIT lumen upon blood circulation and exposure of the liver. Similarly, Falconer [91] reported lesions in the liver and the small intestine upon oral administration of microcystin-containing water to pigs. Furthermore, not only epithelial lesions but also decrease in intraepithelial lymphocytes in the intestine was shown in a murine model [92]. Moreover, changes in the activity of membrane enzymes and in the peroxidation status in rat intestine was proven [93].

Besides uptake from the GIT, lesions of the small intestine (i.e. the jejunum) have been reported in mice upon intraperitoneal exposure, indicating toxic mechanisms of the MC-LR excretion into the GIT [90].

In order to better mimic the human GIT and to account for interspecies extrapolation, a limited number of experiments on human gut-derived cell lines, especially the human colon carcinoma-derived cell line CaCo-2, that exhibits properties of the small intestine upon *in vitro* differentiation, were conducted [94]. These studies confirm a rapid uptake of MC-LR in apical-basolateral direction over a CaCo-2 monolayer [95] and indicate the susceptibility of intestinal epithelia to MC toxic effects, as observed in afore-mentioned *in vivo* studies [90]. Among others, cytotoxicity of microcystins is accompanied by oxidative stress (lipid peroxidation induction, reactive oxygen species production, reduced glutathione content etc.) and cytokine production [96]. Interestingly, MC-LR induced the same levels of IL-6 but much higher levels of IL-8 than MC-RR in CaCo-2 cells after 24 h [97]. Transcriptomic analysis showed quite similar profiles induced by MC-RR and MC-LR but overall gene expression was higher in the case of MC-LR [98]. Interestingly, the uptake of these two congeners does not differ in CaCo-2 cells and both of them are able to enter the nucleus [99]. Further, microcystin cytotoxicity could depend also on the presence of more hydrophobic amino acids in the molecule. It was published that the congeners MC-LF and MC-LW displayed higher

246 cytotoxicity in CaCo-2 cells in comparison to MC-LR [100]. Zegura et al. even observed a comparable
247 sensitivity of CaCo-2 and hepatic HepG2 cells to MC-LR exposure [101].

248 Microcystin also plays a role in neoplastic transformation in intestinal cells. It constitutively activates
249 Akt and p38, JNK and MAPK pathways in immortalized colorectal crypt cells which leads to their
250 proliferation and anchorage-independent growth phenotype [76]. MC-LR increases migrative and
251 invasive potential of HT-29, DLD-1 and SW480 human colon cell lines increasing matrix
252 metalloproteinase-13 expression [102]. Higher migration and invasion of HT-29 cells after MC-LR
253 treatment is also connected with increase in cadherin-11 expression [103]. MC-LR also decreases E-
254 cadherin expression and increases expression of Vimentin and Snail in DLD-1 and HT-29 cells
255 promoting epithelial-mesenchymal transition [104]. Very new publication shows MC-LR ability to
256 contribute to migration of DLD-1 cells via changes in microRNA-221 expression and STAT3
257 phosphorylation [105].

258 In addition to the liver, the intestines should also be considered a target of MC toxicity [101,106].
259 Besides acute effects on animals and human colon-derived cell lines, the intestines can be dietary
260 exposed to low doses of MCs, so chronic exposure may adversely affect intestinal tissue [101]. This
261 is of special importance considering a reported tumor promoting and neoplastic transformation
262 potential of MCs [74–76,81,107].

263 **Table 2: Overview of microcystin (MC) effects on the gastrointestinal tract and/or the (gut-associated) immune system.** CYN: cylindrospermopsin; EC50: Half-maximal effective
264 concentration; ERK: extracellular signal-regulated kinase; LD50: half-maximal lethal dose; LOAEL/NOAEL: Lowest/no observed adverse effects level; LOEC/NOEC: lowest/no observed
265 effect concentration; MAPK: mitogen-activated protein kinase; MC: microcystin; OATP: organic anion transporting peptide; NF-κB: nuclear factor κB; PP2A: protein phosphatase 2A; s.p.f.:
266 special pathogen-free; TEER: trans-epithelial electric resistance.

| Experimental Model | Assays performed, endpoint | Exposure conditions, Concentration ranges | Affected Tissue(s) of interest | Main Results | Reference |
|---|---|---|---|---|---|
| *In vivo* | | | | | |
| Mouse (ICR, female) | Radionuclide recovery | 3H-dihydro MC-LR, 70 µg/kg bw (i.p.); detection after 3-90 min | Stomach, small intestine, large intestine, gastrointestinal tract | Detection of exposure-linked radioactivity in the gastrointestinal tract (approx. 37.6% of the total administered dose), especially in small intestine (6.4 %) | [5,108] |
| Mouse (aged Balb/C, ICR) | Immunohistochemistry | MC-LR, 500 µg/kg bw (p.o.); 1-13 weeks | Stomach, small intestine, caecum | Especially small intestine (villi and *lamina propria*) stained highly immunopositive; erosion of small intestine | [88] |
| Mouse (Balb/C, s.p.f., 7-week old female) | Histology, immunohistochemistry | 75% of MC-LR LD50 (i.p.), 8-32 h | Small intestine | Apoptotic indices after 32 h exposure: 4.25 ± 0.125% (duodenum), 2.5 ± 0.125% (jejunum), 1.75 ± 0.125% (ileum) | [90] |
| Mouse (N:NIH-S, male) | Phosphatase inhibition assay | MC-LR, 50 µg/kg bw (p.o.) every 48 h, 30 days | Gut-associated lympohoid tissue | Decrease of intraepithelial lymphocytes by 28.7 % ± 5.0% | [92] |
| Mouse (Swiss albino, female) | Comet assay (single-cell gel electrophoresis; DNA-damage) | 10 mL/kg bw (p.o. or i.p.), 3-24 h. oral doses: 2-4 mg MC-LR/kg bw; i.p. doses: 10-50 µg MC-LR/kg bw. | Blood, liver, kidney, small intestine (ileum), large intestine (colon) | DNA damage induced in intestinal tissues (ileum and colon) may contribute to increased cancer risk | [109] |
| Rat (Wistar) | Determination of MC-LR toxicokinetics by histopathology and LC-MS detection | $MC\text{-}LR_{equivalent}$, 80 µg/kg bw (i.v.); 1-24 h | stomach | Detection of MC in different tissues upon intravenous gavage; 0.010–0.058 µg/g dry weight $MC\text{-}LR_{equivalent}$ in the stomach | [110] |
| Fish (medaka) | Immunohistochemistry | 5 µg/g MC-LR bw (p.o., direct administration to the fish stomach), 2 h | Gut-associated lymphoid-tissue, intestine (submucosa) | MC-positive staining of submucosa (penetration through the epithelium); disrupted cellular cohesion; MC-positive stained macrophages | [111] |
| Human (fishermen) | Epidemiology, cohort study, risk assessment | $MC\text{-}LR_{equivalent}$ | Whole organism | Estimated daily intake: 2.2-3.9 µg;<br>LOEL, tolerated daily intake: 0.28 µg/kg bw/day | [40,91] |
| *In vitro* | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| Human (CaCo-2) | Apparent permeability of the pseudoepithelial cell layer to MC-LR | 1-75 µM MC-LR; 0.5-24 h | Intestine (colon) | Apical-to-basolateral transport: 24-40% decrease in apical compartment/0.3-1.3% increase in basolateral compartment; low efflux from cellular to basolateral compartment. basolateral-to-apical transport: slow concentration decrease (basolateral, fast increase (apical); better efflux in basolateral-to-apical direction | [95] |
| Human (CaCo-2) | Immunolocalization (microcystin uptake) | 1-50 µM MC-LR, -RR, 0.5-24 h | Intestine (colon) | Rapid uptake in less than 1 h of both variants (no difference in uptake profile); nuclear localization of MCs upon uptake; facilitated uptake (probably via OATPs) and active excretion | [112] |
| Human (CaCo-2) | Gene expression, transcriptomics | 10-100 µM MC-LR, 4-24 h | Intestine (colon) | Major effects on oxidative stress, ERK/MAPK and cell cycle pathways | [98] |
| Human (CaCo-2) | Bradford assay (Cell number, protein content), neutral red uptake, MTS reduction (viability) | MC-LR, -RR, -YR; 50-200 µM, 24-48 h | Intestine (colon) | EC50: reduction of total protein content: 111.1 ± 3 µM MC-LR (24 h), >200 µM MC-RR (48 h); neutral red uptake: 57.3 µM MC-YR (48 h) | [106,113] |
| Human (CaCo-2) | MTT assay, Comet assay | 0.2-10.1 µM MC-LR, 4-48 h | Intestine (colon) | 40% reduced cell viability upon 48 h exposure to 10 µM MC-LR (MTT assay), 19.6% damaged DNA after 4 h exposure to 0.2 µM MC-LR | [101] |
| Human (CaCo-2) | Lactate dehydrogenase (LDH) leakage (cytotoxicity), cell proliferation and morphology, Protein phosphatase (PP) inhibition | 1-50 µM MC-LR, -LF, LW, 22-48 h | Intestine (colon) | EC50: LDH leakage: 25 % (50 µM MC-LR, control), 36 % (MC-LW), 51 % (MC-LF); PP inhibition: 3.0 nM MC-LF, 3.8 nM MC-LW, 1.0 nM MC-LR; apoptosis and morphological changes: membrane blebbing, cell shrinkage, chromatine condensation, cytoskeletal reorganization | [114] |
| Human (IEC-6) | CCK-8 (cell viability), apoptosis, transepithelial electric resistance (TEER), PP2A activity, western blot | 0-50 µM MC-LR, 6-24 h | Intestine (colon) | LOEC: TEER:50 µM (12 h), 12.5 µM (24 h); viability: 12.5 µM (24 h); apoptosis: 25 µM (24 h); western blot: 12.5 µM (24 h; occludin), 25 µM (24 h; ZO-1); PP2A activity: 12.5 µM (24 h) | [115] |

| | | | | | |
|---|---|---|---|---|---|
| Human (NCC) | Genechip analysis, western blot, kinase activity assays, proliferation | 0.1-1005 µM MC-LR, 28 d | Intestine (colon) | Transformation (first step in carcinogenesis); constitutive upregulation of signaling pathways (PI3K, APK2, Akt, cyclin D1 and D3), of Ras GTP/GDP proteins (IQGAP-2, RabGTPase , Rap1GAP , RasGAP , R-Ras , Krev-1, TC21) and Pas/MAPK pathway (A-Raf, B-Raf, PAK); decreased proliferation of MC-LR-transformed colorectal crypt cells | [76] |
| Human (DLD-1, HT29) | Western blot, RT-qPCR, gene knockdown by siRNA, cell migration | 0.1-50 nM MC-LR, 24 h | Intestine (colon) | Motility acquired by epithelial-mesenchymal transition through exposure to 25 nM MC-LR (LOEC) in both colorectal cancer cell lines; MC-LR is likely to aggravate (colorectal) cancer development | [74] |
| Mouse (Balb/C, isolated peritoneal macrophages) | mRNA expression | 1-1000 nM MC-LR + 100 µg/L LPS; 6 h | Innate immune system | Reduction/alleviation of LPS-induced inflammation | [116] |
| Mouse (RAW 264.7, blood macrophages) | Western blot, ELISA | 1-1000 nM MC-LR, 0.5-24 h | Innate immune system | LOECs upon 24 h exposure to MC-LR for: MAPK (ERK1/2) activation (100 nM), NF-kB activation (1000 nM), TNFa production (1 nM) | [68] |
| Human (predominantly) | Case study meta-analysis, review | | | References to human intoxication cases, relation between oesophagus cancer and human contact with cyanotoxins through the food chain requires further investigation | [12] |

## Cylindrospermopsin effects

Cylindrospermopsin (CYN), a tricyclic guanidine alkaloid was first characterized in *Cylindrospermopsis raciborskii* [117] upon a major intoxication event in Palm Island, Australia [15]. Despite the still not entirely elucidated molecular mode of action, CYN is proven to be a potent inhibitor of protein synthesis (LOEC = 0.5 µM CYN, primary mouse hepatocytes) [5,118,119].

In addition, CYN has been found to induce tissue damage, cytotoxic effects, oxidative stress and DNA damage in a variety of organs and cell types [118].

CYN toxicity was reported to be attenuated by different inhibitors of cytochrome P450 (CYP450) *in vivo* [120], as well as in the cultured cells *in vitro* [121–124] [125,126]. Induction of CYP450 activity also increased CYN toxicity in hepatic cell lines [119,125,127]. Thus, it has been proposed that CYP450 activity is responsible for CYN bioactivation, increasing especially CYN-induced acute cytotoxicity, oxidative stress and genotoxicity, while inhibition of protein synthesis appeared to be unaffected and attributed to the parental compound [119,128]. However, no biotransformation products of CYN have been detected upon incubation with S9 liver fraction or in the presence of metabolically-competent liver cell lines [126], and also S9 liver fraction was not found to increase genotoxic potential of CYN in other studies [129,130]. Thus, the exact role of CYP450 in CYN toxicity is not completely clear and should be investigated in the future [118]. Phase II detoxification of CYN is supposedly mediated via glutathione conjugation and excretion in the liver and kidney [5,118,122,131,132]. These detoxifying and excreting organs, liver and kidney, are also the most sensitive tissues (NOAEL = 30 µg/kg/day; in mice) and the major recipients of CYN toxicity, but adverse effects on the stomach, the small intestine and on white blood cells have also been reported [133–135].

Due to the hydrophilic zwitterionic nature of CYN, similarly to MCs, it probably cannot be readily absorbed from the gastrointestinal tract upon ingestion, the main pathway of human exposure, hence, it has been hypothesized to require a facilitated or active transport [136,137]. Exposure of mice to CYN induced ulcers in the stomach and bleeding into the stomach and small intestine (2.5-8.3 mg $CYN_{equivalent}$/kg, 2-8 d; Table 3) [135]. Nevertheless, there is a lack of research addressing the gastrointestinal effects of CYN. Only two studies on colon-derived tumor cells (CaCo-2) address

296 uptake kinetics across the intestinal epithelium, both reporting only a limited passage of the toxin
297 through the intact epithelium [94,138]. Pichardo et al. [138] furthermore conclude, that paracellular
298 diffusion is the most likely uptake mechanism of CYN from the intestinal lumen, while active
299 transport via P-glycoprotein or multidrug resistance proteins probably contributes to the secretion
300 and elimination of CYN. Despite the observed uptake across the epithelial barrier of intestinal cells
301 *in vitro*, CYN causes oxidative stress and decreases cell viability in CaCo-2 cells in a dose-dependent
302 manner (see Table 3), highlighting that enterocytes (as well as hepatocytes) also should be
303 considered a major important recipient of CYN toxicity, especially upon oral exposure [139].

**Table 3: Overview of cylindrospermopsin (CYN) effects on the gastrointestinal tract and/or the (gut-associated) immune system.** CYN: cylindrospermopsin; EC50: Half-maximal effective concentration; GSH: glutathione; IL: interleukine; LOAEL/NOAEL: Lowest/no observed adverse effects level; LOEC/NOEC: lowest/no observed effect concentration; MC: microcystin; ROS: reactive oxygen species; TDI: tolerated daily intake.

| Experimental Model | Assays performed, endpoint | Exposure conditions, Concentration ranges | Affected Tissue(s) | Main Results | Reference |
|---|---|---|---|---|---|
| *In vivo* | | | | | |
| Mouse (MF1, male) | Histology | *C. raciborskii* culture extract containing 0.2% CYN; 2.5-8.3 mg/kg $CYN_{equiv.}$ (gavage), 2-8 d | Esophagus, stomach, small intestine | Stomach ulceration, fresh blood in (small) intestinal content | [135] |
| Mouse (Swiss albino) | Body and organ weight, urine, serum, hematology analysis, histopathology | CYN-containing cyanobacterial extract, 0-657 µg CYN/kg/day (p.o.), 10 weeks; purified CYN, 0-240 µg CYN/kg/day (p.o.), 11 weeks | | NOAEL (TDI): 30 µg/kg/day; proposed guideline value for drinking water: 1 µg/L | [140] |
| *In vitro* | | | | | |
| Human (CaCo-2) | Cytotoxicity (Neutral Red uptake) | C. raciborskii (CYLI29, CYN/MC-free methanolic extract), C. raciborskii (AWT205; 1.1 mg CYN/g dw; methanolic extract); 0.08-1.25 mg dw/mL, 48 h | Intestine (colon) | EC50: 0.4 ± 0.1 mg dw/ml (CYLI29), 1.3 ± 0.2 mg dw/ml (AWT205) | [141] |
| Human (CaCo-2) | Intestinal permeability, epithelial integrity (trans-epithelial electric resistance, TEER) | 1-10 µM CYN; 3-24 h | Intestine (colon) | 16.7-20.5% (intestinal permeability, apical-to-basolateral, after 24 h), epithelial integrity not significantly altered | [94] |
| Human (CaCo-2) | Protein content (Bradford assay), cell viability (MTS reduction), oxidative stress, intracellular GSH content, ultrastructural alteration | 0.72-96.3-µM CYN 24-48 h | Intestine (colon) | LOEC: 1.44 µM (cell viability, ultrastructural alteration), 3.0 µM (intracellular ROS concentration), 6.0 µM (protein content, GSH content) | [139] |
| Human (CaCo-2, C3A, HepG2, NCI-87, HCT-8, HuTu-80) | Cell viability (MTT assay) | 0.25-5 µM CYN, 1-7 d | Intestine (colon), liver, stomach, small intestine (ileus, duodenum) | cell-line sensitivity decreased in cell lines derived from more distal regions of the gastrointestinal tract: gastric > duodenal > ileal > colonic; EC50 = 6.5 ± 3.3 µM (CaCo-2) | [142] |

| | | | | | |
|---|---|---|---|---|---|
| Human (CaCo-2) | Permeability of pseudoepithelial layer | 1.9-48.1 µM CYN, 24-48 h | Intestine (colon) | Apparent permeability: 3.45 × 10−7 cm/s (absorptive direction), 6.41 × $10^{-7}$ cm/s (secretive direction); epithelial permeability (increase): 10-fold (absorptive), 0.7-fold (secretive); negligible transcellular passage | [138] |
| Human (primary lymphocytes, whole blood) | T-lymphocyte proliferation (thymidine incorporation) | 1% biomass extract, 72 h (lymphocytes), 0.24 nM CYN, 72 h (whole blood) | Blood, immune system | Significant reduction of T-lymphocyte proliferation | [143] |
| Human (primary peripheral blood neutrophils) | Oxidative burst capacity (NADPH oxidase mediated) | 0.024-2.4 µM CYN, 1 h | Blood/innate immune system (neutrophils) | Significantly decreased ROS production at all CYN concentrations tested (LOEC = 0.024 µM CYN) by decreased NADPH oxidase-mediated ROS production in neutrophils; phagocytic activity unaffected | [144] |
| Fish (*Cyprinus carpio*, isolated phagocytic cells) | IL-1b expression (PCR, phagocytosis, oxidative stress | 0.12-2.4 µM CYN, 24 h | Immune system | 32-fold increase in IL-1b expression (2.4 µM, 24 h); diminished phagocytosis (1.2 µM), ROS-production increased (0.12 µM; LOEC) | [145] |

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309 ## Effects on the immune system

310 Besides reports of effects on gastrointestinal and hepatic cells, MC-LR is suspected to alter the
311 immune response, especially in fish, where most of the studies addressing immunotoxic effects have
312 been done [111,146–150]. For example, exposure to MC-LR affected the immune response of
313 medaka fish additionally to sustained pathological changes in the GIT, liver and other organs [111].
314 In a mammalian (mouse) *in vivo* model, MC-LR has been shown to decrease levels of intraepithelial
315 lymphocytes, thus affecting mucosal immunity [151]. *In vitro* exposure of murine macrophages to
316 MC-LR significantly altered the expression of pro-inflammatory genes and the release of cytokines
317 (Table 2) [68,116]. Adamovsky et al. [68] additionally proposed a receptor (TLR)-mediated
318 mechanism of macrophage activation, as their model lacked specific transmembrane transporters,
319 hence, the common mode of MC-LR action cannot be taken into regard.

320 Similarly to MC-LR, CYN exposure led to elevated markers of inflammation and diminished the
321 uptake of bacteria into phagocytes in the common carp (*Cyprius carpio*) [147]. Although there are
322 significant differences between immune systems of different groups of animals, vertebrates share
323 some common mechanisms of innate immune system activation. These first-line defense
324 mechanisms are, amongst others, increased expression of pro-inflammatory cytokines (e.g. IL-6 and
325 TNFα), production of reactive oxygen species (ROS), secretion of nitric oxide and phagocytosis
326 [68,152–154]. We recently discovered that CYN activates murine macrophages *in vitro* (unpublished
327 data). In addition, CYN (0.1 µg/L; 0.24 µM) has been shown to significantly decrease lymphocyte
328 proliferation *in vitro* in both isolated T cell culture and a whole-blood assay [143].

329 The effects on the immune system from *in vivo* studies are often evaluated as morphologically
330 altered lymphoid follicles, changes in spleen size or weight, but the underlying mechanisms are not
331 investigated or discussed any further [118,135].

332 Even though these findings have to be elaborated on and verified in a human health relevant system,
333 disturbances in these fundamental immunological processes may lead to systemic effects that are
334 of special concern when environmentally relevant mixture effects or effects on sensitive people with

more pronounced immune reactions are evaluated and should be considered for future risk assessments.

## Lipopolysaccharide effects

Like most of the commensal and pathogenic bacteria, cyanobacteria stain Gram-negative. This group of microbes shares a common structural feature: they incorporate lipopolysaccharides (LPSs) in their cell wall. Eubacterial LPS is known as a model ligand of the Toll-like receptor 4 (TLR4), a member of pattern recognition receptor (PRR) family expressed on epithelial, endothelial, immune (particularly macrophages and dendritic cells) and other cells being an important part of the innate immunity [155–157]. In order to maintain homeostasis in exposed tissues, activation of PRRs may launch a robust immune reaction, eventually preventing the invasion of microbes into other tissues of a healthy individual [158,159].

LPS binding to the TLR4-MD-2 receptor complex may initiate MyD88-dependent signaling, resulting in the production of pro-inflammatory cytokines (e.g. IL-6, IL-8, IL-12, TNFα), or in MyD88-independent signaling causing the release of type I interferons. Both pathways lead to activation of the transcription factor NF-κB, governing the expression of pro-inflammatory cytokines [155,160,161]. Even though LPS is traditionally considered one of the most potent pro-inflammatory agents, there is an increasing evidence that structural variation, i.e. the degree and site of acylation of the immunogenic lipid A moiety alters the immunogenicity of LPS, which may even elicit anti-inflammatory properties [162–165].

Despite the probable oral exposure to cyanobacterial LPS (cyanoLPS) in bloom biomass, this topic has received little scientific attention. Compared to LPS from heterotrophic bacteria, cyanoLPS differs in structure. For example, lipid A of LPS produced by *Anabaena* spp. lacks phosphorylation and glucosamine, while being acylated at up to ten sites [166–169]. Altered acylation of LPS significantly influences the magnitude of LPS inflammatory potential with hexaacylation triggering the strongest inflammatory responses [162,170].

In the gastrointestinal epithelial layer, constantly exposed to a large variety of LPSs, the expression of functional TLR4 is low. Phagocytic cells of the innate immune system expressing TLR4 are rather found in the submucosal *lamina propria* [170–172]. Subsequently, the immune system in healthy

individuals recognizes and scavenges only bacteria invading the GIT tissue by crossing the mucosal/epithelial barrier (Munford 2008 and references therein).

Nevertheless, the first and most widespread human acute health effect upon (accidental) ingestion and intoxication of cyanobacterial bloom material is gastrointestinal illness, often with severe inflammatory diarrhea (enterocolitis) [6,12,13,173]. A prime suspect for this common symptom of cyanobacterial oral exposure is LPS.

Regardless of the strong rationale for cyanoLPS acting as a mediator of severe gut inflammation upon oral exposure, experimental evidence is less conclusive. Compared to bacterial (*Escherichia coli* K12) LPS standards, the pro-inflammatory potency of isolated cyanoLPS was approximately 10-fold lower or protective to *E. coli* LPS-induced inflammation [164,166,174–176]. The reduced ability of cyanoLPS to mount a robust inflammatory response compared to LPS produced by heterotrophic bacteria may be attributed to different lipid A structures and restrictions in the molecular pattern recognized by the cellular receptor [170]. Interestingly, Stewart et al. identified even a lack of evidence to support gastrointestinal pro-inflammatory reaction of heterotrophic bacterial LPS alone, in the absence of other virulence factors [175,177]. Considering the pro-inflammatory potential of bloom extracts, including bloom-associated heterotrophic bacteria, a cumulative action of several (cyano-) bacterial components is very likely [166,174–176,178]. For example, LPS may contribute to local GIT tissue inflammation and thereby facilitate the access of other toxic compounds, like MCs, to deeper tissues and distribution to other organs and targets of toxicity such as the liver [178].

### Other toxins

Nodularins (NODs) are cyclic non-ribosomal pentapeptide toxins, produced almost exclusively by *Nodularia spumigena*, that share structural and mechanistic similarities with MCs: non-proteinogenic amino acids like N-methyldehydrobutyrine and D-erythro-β-methylaspartic acid are incorporated into the peptide ring structure and the ADDA residue mediates toxicity [5 and references therein]. *N. spumigena* favors slightly saline environments, therefore besides MCs, NOD is a major concern in the Baltic Sea and other brackish habitats [179,180]. There are little data available on NOD toxicokinetics, but an uptake mechanism (OATP) and mode of action (PP1 and

PP2A inhibition) similar to MCs is proposed and partly supported by experimental evidence in zebrafish and different hepatic cell lines [5,181,182]. Also, NOD has an experimental LD50 of 50-70 µg/kg bw in rodents, which is similar to that of MC-LR (40 µg/kg bw) [2,5]. Therefore, NODs can be expected to cause adverse effects on the GIT similar to MCs. Despite severely inhibiting activity of protein phosphatases, NOD in contrast to MCs does not bind covalently to these ubiquitous cytoplasmic enzymes [183].

The cyanobacterial neurotoxins anatoxin-a, anatoxin-a(S) and saxitoxin are fast actors, leading to paralysis and eventually respiratory failure within minutes to few hours [184,185]. Due to the rapid onset and specificity of neurological symptoms induced by inhibiting the transfer of excitatory signals, gastrointestinal irritation is unlikely to be a primary effect of these toxins. Nevertheless, exposure by complex cyanobacterial blooms will probably be to a mixture of many cyanobacterial and bacterial components that may very well result in enterotoxic effects of other metabolites (see also Table 4).

Except of the well-recognized cyanotoxins, there are many potentially harmful yet poorly characterized bioactive secondary metabolites. Many of these, like the non-ribosomal peptides aeruginosins, anabaenopeptins or cyanopeptolins, show protease inhibiting activities, the latter has been also shown to cause neurotoxic effects and alter pathways linked to DNA damage and repair (Table 4) [186–191].

Another group of poorly characterized cyanobacterial secondary metabolites are cyclic lipopeptides. Representatives of this group are for example anabaenolysins and puwainaphycins, causing damage to eukaryotic cell membranes also in GIT models and inducing necrotic effects (Table 4) [192–194]. More explicit effects on the GIT are reported by Humpage et al. (2012). They described necrotic effects of the novel putative toxin “limnothrixin”, isolated from *Limnothrix* spp., upon oral gavage of an aqueous extract, in liver, kidney and GIT tissue within 24 h of exposure (Table 4). Even though no known toxin was detected in the aqueous extract, there may still be a variety of hydrophilic compounds present and contributing to the effects observed.

The toxicological implications of these activities remain to be elucidated and the characterization of novel putative toxins in an effect-directed screening approach along with the description of

419 structural characteristics should be highly encouraged. Regardless of the unknown toxic potential,
420 these substances may contribute to the severity of gastroenteritis upon cyanobacterial intoxication,
421 for example by facilitating enteric hemorrhage through interference with the blood coagulation
422 cascade [187].

**Table 4: Overview of other (non-MC, non-CYN) cyanobacterial metabolites' effects on the gastrointestinal tract and/or the (gut-associated) immune system**. CyP: cyanobacterial LPS-like compound; EC50: half-maximal effective concentration; ELISA: enzyme-linked immunosorbent assay; GIT: gastrointestinal tract; IFNγ: Interferone γ; IL: interleukine; LAL: *Limulus* amebocyte lysate; LOAEL/NOAEL: Lowest/no observed adverse effects level; LPS: Lipopolysaccharide; PUW: puwainaphycin; TLR: toll-like receptor.

| Compound | Experimental Model | Assays performed, endpoint | Exposure conditions, Concentration ranges | Affected Tissue(s) | Main Results | Reference |
|---|---|---|---|---|---|---|
| | *In vivo* | | | | | |
| Cyanobacterial LPS | Mouse (C57BL, female) | Mortality | 0.025-1.5 mg bacterial LPS (i.p.), co-injection of 750-850 µg CyP | Whole organism | Protection against LPS-induced septic shock after 8 h (25 ng LPS; 58% survival, D-galactosamine-sensitized mice) and after 24-40 h (1.5 mg, 80% survival, non-sensitized mice) | [165] |
| "Limnothrixin" (from *Limnothrix* AC0243) | Mouse (Balb/C, male) | Histology | 300 µl known-toxin-free biomass extract (i.p.; extract 1: 180 mg dw/mL, extract 2: 195 mg dw/mL), 3-24 h | Gastrointestinal tract, small intestine (duodenum) | Loss of single cells/cell sheets in the duodenum; serum-colored mucoidal material in the gastrointestinal tract 3-10 h p.i. | [195] |
| *C. raciborskii* biomass extract without detectable levels of CYN, MCs or saxitoxins | Mouse (Charles River, male) | Histology | 1337-1572 mg dw/kg bw (i.p.), 8-24 h | Intestines, immune system (mucosa-associated lymphoid tissue) | Enlarged Peyer's patches | [196] |
| | *In vitro* | | | | | |
| Aeruginosins | | In vitro inhibitory assays (biochemical) | | | Serine protease inhibition | [187] |
| Anabaenolysin A | human (primary erythrocytes) | Haemoglobin release (necrosis) | 0.38-3 µM Abl A; 1 h | blood | EC50 (necrosis) ≈ 0.8 µM | [192] |
| Cyanobacterial LPS | Human (primary monocytes) | ELISA, RT-PCR, FACS analysis | 0.1-20 µg/mL cyanobacterial LPS + 1-10 µg/mL bacterial LPS; 0-16 h | Blood (dendritic cells), innate immune system | 20-fold excess of CyP compared to LPS completely inhibited cytokine production | [165] |
| Cyanobacterial LPS | | Pyrogenicity (LAL test, PyroGene rFC assay), leukocyte activation (chemiluminescence) | Cyanobacterial LPS-extracts from *M. aeruginosa*-dominated bloom-biomass (11 naturally occurring blooms) | Immune system | 10.2-78.3×104 EU/mg LPS (LAL test) ; 0.91-18.96×$10^4$ EU/mg LPS (PyroGene assay) ; leukocyte activation observed | [174] |

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|---|---|---|---|---|---|---|
| Cylindrospermopsin-containing biomass extracts | | Skin sensitation | Human health risk assessment, literature review | Skin, immune system | Skin sensitation occurred in a cylindrospermopsin-independent manner, implying other irritating agents in the bloom biomass | [178] |
| Cyanobacterial biomass (*Spirulina platensis*) hot water extract | Human (blood, male volunteers) | Cytokine production and responsiveness, natural killer cell cytolitic activity | 50 mL extract/volunteer/day, 1-8 weeks | Whole organism, blood | Internalization of yet uncharacterized *Spirulina* components via GIT; prestimulation of monocytes. Targets: monocytes (additive effect on TLR-mediated cytokine production) and natural killer (NK) cells (upregulation of cytolysis and IFN; critically affected by IL-18 levels). | [197] |
| Cyanopeptolin CP1020 | *Danio rerio* (whole embryo) | transcriptomics | 0.1-1 mg/L CP1020, 96 h | Whole embryo | Pathways altered<br>≥2-fold: DNA damage recognition and repair, circadian rhythm, response to light. | [190] |
| Puwainaphycin F | Human (CaCo-2) | ELISA | non-cytotoxic concentrations of PUW1146, PUW1118, PUW1188; 24 h | Intestine (colon) | Increased secretion of IL-8, altered expression of tight junction protein | [194] |
| Puwainaphycins F/G | Mouse (YAC-1 cells), human (HeLa) | MTT assay, intracellular $Ca^{2+}$ concentration | 1-10 µM PUW, 10 min - 10 h | lymphoma, cervical cancer | EC50 (necrosis) 2.2 µM | [193] |

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## Co-action of different factors on the GIT

While single-agent toxic effects are often reported for detoxifying organs like the liver or the kidneys in toxicological studies, the most prominent symptom of harmful algal bloom-intoxication is enterocolitis, probably mediated by coaction of a multitude of virulence factors. Besides the occasionally high abundance of already identified cyanobacterial toxins like MCs, many of the secondary compounds produced by cyanobacteria and eventually released to water are poorly characterized concerning their toxicity or not identified yet (Table 4) [178,196,197]. Furthermore, bloom-associated bacteria may contribute to the adverse effects observed upon exposure to cyanobacterial blooms, especially gastrointestinal illness [43,198]. The complex composition of cyanobacterial blooms also leaves space for additive or even synergistic effects of the multitude of compounds, which may exacerbate the impact of exposure to otherwise moderately active/toxic compounds.

Also the role of LPS of both, cyanobacterial and eubacterial origin has to be critically reflected. The controversies about pro- or anti-inflammatory activities of cyanoLPS may be explained by effects of bloom associated bacterial LPS, potentially sensitizing GIT lining epithelia for the effects of other toxins. A similar effect was observed with the pore-forming lipopeptides anabaenolysins A and B, where a transient increase in cell membrane permeability facilitated nodularin uptake, lowering the effective concentration for nodularin toxicity [192]. CyanoLPS activity on the (humoral) immune system does not sufficiently explain the GIT symptoms observed upon acute oral exposure to cyanobacterial blooms. But total bloom-LPS, including pro-inflammatory LPS from bloom-associated bacteria, may facilitate the penetration of gastrointestinal epithelia and thereby promote the uptake of other cyanobacterial toxins by macrophages or into the blood via the paracellular route as suggested for CYN [138,175].

Considering the described activity of MC-LR as a tumor promoter and CYN being suspected of carcinogenicity, the mixture of substances diverse in biological activity could finally also contribute to colorectal cancer incidents upon long-term exposure to low concentrations e.g. in drinking water [21,74,76,199].

## Conclusion

Cyanobacterial blooms are occurring more frequently and in increasing severity due to global climate change and eutrophication of water bodies, endangering the recreational value of water bodies as well as the safety of drinking water supplies. Hazards linked to cyanobacterial contamination have been recognized and addressed by regulatory authorities (WHO, EFSA, EPA). For cyanobacterial bloom management, the precautionary principle is proposed, that means the bloom is considered hazardous until proven safe [186].

Despite gastrointestinal symptoms being the most reported and wide-spread malaise upon oral exposure to cyanobacterial bloom biomass, research mostly focuses on specific organ toxicity of isolated toxins, i.e. hepatotoxic MCs and CYN. The oral route remains the most relevant exposure route for humans and gastrointestinal distress the predominant symptom. Consequently, the gut epithelia are exposed to the highest toxin concentrations and are also the first barrier that needs to be overcome for the toxins to reach the blood stream and be subsequently distributed to other organs/recipients of toxicity like the liver. In the naturally occurring complex mixture of cyanobacterial bloom material, a multitude of factors can contribute to the adverse effect observed on the GIT, probably not attributable to a single toxin or agent. Nevertheless, even isolated toxins are reported to adversely affect the (small) intestine (MC-LR, CYN) or the stomach (CYN), highlighting the importance of further investigation of this neglected yet plausible and relevant system. MC-LR, for which the scarce evidence on GIT irritation is strongest, should even be reconsidered as an enterotoxin.

Taking into consideration sensitive subpopulations, children and people with chronic gastrointestinal inflammations (e.g. Crohn's disease) are at higher risk. They suffer more often and from more severe enteritis, as their humoral immune system is still under development or in a permanently inflamed state. Also, with regard to the carcinogenic and genotoxic potential of MCs and NODs, low-level chronic exposure may contribute to colon carcinoma in later years [134,173,200,201].

## List of abbreviations

Adda: (all-*S*,all-*E*)-3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; ATX: anatoxin, EC50: Half-maximal effective concentration, EFSA: European Food Safety Agency, EPA: Environmental Protection Agency, GIT: gastrointestinal tract, IL: interleukine, LD50: half-maximal lethal dose, LOAEL/NOAEL: Lowest/no observed adverse effects level, LOEC/NOEC: lowest/no observed effect concentratio, LPS: lipopolysacharide, MAPK: mitogen-activated protein kinase, MCs: microcystins, NF-κB: nuclear factor κB, NOD: nodularin, OATP: organic anion transporting peptide, PAMP: pathogen-associated molecular pattern, PP1/2A: protein phosphatase 1/2A, PRR: pattern recognition receptor, ROS: reactive oxygen species, STX: saxitoxin, TEER: trans-epithelial electric resistence, TLR: toll-like receptor, TNFα: tumor necrosis-factor α, WHO: World Health Organization.

## Declarations

a. **Availability of data and material:** Not applicable.

b. **Competing interests:** The authors declare no conflict of interest.

c. **Authors' contributions:** Conceptualization, K.H., P.B., L.Š. and B.K.; methodology, B.K.; writing—original draft preparation, B.K.; writing—review and editing, K.H., P.B., L.Š. and B.K.; supervision, K.H. and P.B.; project administration, K.H., L.Š.; funding acquisition, K.H., L.Š."

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## Materials and Methods, Description of workflow

In order to obtain relevant scientific literature, a systematic review of publications was performed according to the PRISMA scheme (Preferred Reporting Items for Systematic reviews and Meta-Analyses; online supplementary Figure S1). The Scopus database, including PubMed, Web of Knowledge and Science Direct, was searched for the key words: ( TITLE-ABS-KEY ( cyanobacteria OR cyanotoxins OR microcystins OR bloom* ) AND TITLE-ABS-KEY ( epidemiology OR incidence OR health OR health AND effects OR health AND risk OR health AND impact OR health AND hazard OR adverse AND effects OR risk AND assessment OR exposure OR drinking AND water OR intoxication OR disease OR illness ) AND TITLE-ABS-KEY ( gastrointestinal OR gastro* OR enteric

OR allerg* OR gastric OR inflamm* OR lps OR lipopolysacch* ) . No further limits (Access type, publication date, document type etc.) were defined; all results were in English language (last searched on December 13, 2018). 87 articles were identified through the database search; 55 additional articles were identified by other sources. After removing duplicates (n=16), 126 articles were screened for eligibility based on the abstracts. 44 publications were not eligible on the basis of their abstracts, 82 underwent further full-text assessment. Of these, 66 articles were included for review purposes.

Titles classified as highly relevant (i.e. review articles, epidemiological studies) indicated further sources, not found by searching the Scopus database.

This review elaborates and expands on five review articles identified as highly relevant [4,6–9].

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