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Challenges and opportunities of read-across for the tumor promotion effects of microcystins

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ABSTRACT

The microcystins (MCs) are a family of cyclic oligopeptides toxins expressed in at least 30 cyanobacterial species and are liable to pose significant hazard to human health due to hepatotoxicity. Microcystin-leucine arginine (MC-LR) is the most extensively studied and toxic congener and classified as possibly carcinogenic to humans based on tumor promotion activity in the liver. Given the substantial toxicity data gaps for the MCs, read-across was assessed to evaluate the tumor promotion effects of a series of data-poor MC congeners based on *in vivo* information for MC-LR as the source molecule. Lines of evidence from *in silico* estimates of structural similarity, physico-chemical properties, hepatotoxicity, genotoxic and carcinogenicity were compiled to support the filling of data gaps. Uncertainties were evaluated according to scenario 4 of the European Chemicals Agency's (ECHA's) Read-Across Assessment Framework (RAAF). The read-across process followed a previously proposed harmonized framework to apply the common principles together with information from new approach methodologies (NAMs). Lines of evidence were consistent across the MC congeners and the uncertainties were found to be acceptable for data gap filling. Read-across strategies, with known caveats and restrictions, were shown to be applicable for large, complex molecules such as the MCs.

1. Introduction

Read-across is a data gap filling technique for interpolating or extrapolating biological effects for related chemicals based around the identification of similar analogues holding suitable data to predict an endpoint, or property, for substances which lack empirical data (Cronin, 2013; Kovarich et al., 2019). Whereas read-across was originally based on the extrapolation of information from analogues, there has been a recent shift to compile several lines of evidence in support of similar modes of toxic action and/or metabolic profiles for groups of compounds (Enoch et al., 2022; Gadaleta et al., 2020; Pestana et al., 2021). Information related to the target and source chemical(s) based on non-animal studies, which are referred to as New Approach Methodologies (NAMs) and may include non-guideline studies, has been used increasingly used to support read-across hypotheses (Rovida et al., 2020).

Whilst read-across is commonly applied to small organic molecules and, increasingly, to nanomaterials, it has not frequently been applied to toxins and secondary metabolites. Toxins of natural origin are a group of food contaminants subject to risk assessment by regulatory authorities, e.g. the European Food Safety Authority (EFSA), in order to determine tolerable daily intake (TDI) or the risks following combined exposure to multiple chemicals (Dorne et al., 2021; Cattaneo et al., 2023). Although they are of high interest for hazard assessment purposes, the respective data for toxins are frequently lacking and difficult to obtain experimentally due to the limited accessibility of these substances, opening perspectives to explore read-across approaches.

The microcystins (MCs) are a family of closely-related, cyclic oligopeptides toxins expressed in at least 30 cyanobacterial species (Kianian et al., 2024). MCs are liable to pose significant risk to human health, due to their well-established hepatotoxic potential (Jones et al., 2021). Among this group of congeners, microcystin-leucine arginine (MC-LR) is the most common, most extensively studied and most toxic. It is classified as "possibly carcinogenic to humans", (group 2B) by the International Agency for Research on Cancer (IARC), based on its tumor promotion activity in the liver (IARC, 2010). Exposure, arising typically

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through the ingestion of contaminated water or fish, is commonly influenced by fluctuation in algal population - (IARC, 2010) increasing alongside the occurrence of periodic algal blooms (Testai et al., 2016).

1.1. Challenges of applying read-across to complex molecules such as the MCs

There are few examples of the use of read-across to address data gaps for molecules possessing the structural complexity of the MC family. The challenges associated with read-across to fill data gaps for small molecules (e.g. less than 600 Da) are well documented (Schultz and Cronin, 2017). However, many of these are already adequately addressed (see some of the learnings stated in Roe et al., 2025a,b; Patlewicz et al., 2025; Schmitt et al., 2025) and there are different challenges for larger macromolecules, such as the MCs. From the outset, the following challenges were considered of importance, these could be equally applicable to other molecules of corresponding size and complexity.

- MCs are atypical of the types of molecules where read-across has found success e.g. small, discrete organic molecules, nanoparticles, etc. Therefore, there is less expertise and knowledge on which to base the assessment.
- MCs are challenging molecules for *in silico* analysis, as they are large and conformationally flexible. This may mean that it may be computationally expensive to evaluate them.
- MCs are also inevitably challenging to obtain realistic and meaningful measurements and estimates of their physico-chemical properties.
- Predictions of properties and *in silico* screening must also be used with caution. It is probable that exact values for each MC are unlikely to be accurate as there will be concerns whether the molecules are in the applicability domains. Specifically, MCs may be outside of the applicability domain of many *in silico* models for physico-chemical properties or toxicological effects which have been developed historically from smaller, drug-like, molecules. Therefore, the value of these predictions and estimates is in the relative differences between values for individual MCs, i.e. to demonstrate similarity in terms of a particular property.
- Whilst qualitative read-across may be possible, i.e. the ability to promote tumors, it is more difficult to address quantitative measures of congener potency.

The structural differences in the MCs, particularly exemplified by a wide range of log P values, mean that bioavailability within the group is likely to vary considerably. However, the extent of this variation remains unknown. Given the number of congeners and their importance with regard to human and environmental health, there are very few high quality, relevant *in vivo* data with which to anchor a read-across, when compared to the chemical space breadth of the group.

1.2. Aim of the investigation

Based on the *in vivo* information for MC-LR, a data rich compound, we aimed to develop read-across approaches to fill data gaps for a series of MC congeners. Similarities in chemical structure and a common proposed mode of action were used as the basis for this exercise. Read-across was supported by *in silico* models and techniques characterizing structural similarity, physico-chemical properties, hepatotoxicity, genotoxicity and broader carcinogenicity. Uncertainties were evaluated according to the European Chemicals Agency's (ECHA's) Read-Across Assessment Framework (RAAF) (ECHA, 2017). A harmonized framework was employed as the basis to guide the steps of the read-across process and allowed for the application of common guiding principles together with data from NAMs. The opportunities for success in applying read-across to large, complex molecules were identified.

2. Material and methods: read-across framework applied

Considering that there are 310 MC variants known at the time of undertaking this study, and that this number is likely to increase over time, 17 MCs were selected based on structural similarity and availability of the following information: PP2A inhibition concentration values (IC50) and *in vivo* LD50 (mice intraperitoneal), as collected from literature. MC-LR, a data rich MC, was assigned as the source molecule. Therefore, MCs were grouped on the basis of NAM and *in vivo* data, considering the difference in potency among them. The amino acid compositions of the various congeners selected within this study are listed in Table 1.

The assessment of the read-across for the tumor promotion of MCs was performed by applying and adapting the harmonized hybrid framework developed originally by Patlewicz et al. (2018). The harmonized hybrid framework incorporates the key aspects of read-across from a number of existing frameworks, including regulatory applications and has been adapted as part of EFSA's guidance on read-across (Bennekou et al., 2025). The purpose of using this approach was to provide a means of performing the read-across by compiling relevant data and assessing their validity with as strong a reference to current technical guidance and regulatory applicability in this area as

Table 1
Amino acid composition of the various MC congeners included within this study.

Microcystin congener	Peptide chain position						
	1	2	3	4	5	6	7
MC-LR	D-Ala	L-Leu	D-Masp	L-Arg	Adda	D-γ-Glu	Mdha
MC-RR	D-Ala	L-Arg	D-Masp	L-Arg	Adda	D-γ-Glu	Mdha
MC-LA	D-Ala	L-Leu	D-Masp	L-Ala	Adda	D-γ-Glu	Mdha
MC-YR	D-Ala	L-Tyr	D-Masp	L-Arg	Adda	D-γ-Glu	Mdha
MC-LY	D-Ala	L-Leu	D-Masp	L-Tyr	Adda	D-γ-Glu	Mdha
MC-WR	D-Ala	L-Trp	D-Masp	L-Arg	Adda	D-γ-Glu	Mdha
MC-FR	D-Ala	L-Phe	D-Masp	L-Arg	Adda	D-γ-Glu	Mdha
[D-Asp3,(E)-Dhb7]MC-HtyR	D-Ala	L-Hty	D-Asp	L-Arg	Adda	D-γ-Glu	(E)-Dhb7
[D-Asp3,(E)-Dhb7]MC-LR	D-Ala	L-Leu	D-Asp	L-Arg	Adda	D-γ-Glu	(E)-Dhb7
[D-Asp3,(E)-Dhb7]MC-RR	D-Ala	L-Arg	D-Asp	L-Arg	Adda	D-γ-Glu	(E)-Dhb7
[D-Asp3,ADMAdda5,(E/Z)-Dhb7]MC-HtyR	D-Ala	L-Hty	D-Asp	L-Arg	ADMAdda	D-γ-Glu	(E/Z)-Dhb7
[D-Asp3,ADMAdda5]MC-LR	D-Ala	L-Leu	D-Asp	L-Arg	ADMAdda	D-γ-Glu	Mdha
[D-Asp3,Dha7]MC-LR	D-Ala	L-Leu	D-Asp	L-Arg	Adda	D-γ-Glu	Dha
[D-Asp3]MC-HtyR	D-Ala	L-Hty	D-Asp	L-Arg	Adda	D-γ-Glu	Mdha
[D-Asp3]MC-LR	D-Ala	L-Leu	D-Asp	L-Arg	Adda	D-γ-Glu	Mdha
[D-Asp3]MC-RR	D-Ala	L-Arg	D-Asp	L-Arg	Adda	D-γ-Glu	Mdha
[Dha7]MC-LR	D-Ala	L-Leu	D-Masp	L-Arg	Adda	D-γ-Glu	Dha
[Dha7]MC-RR	D-Ala	L-Arg	D-Masp	L-Arg	Adda	D-γ-Glu	Dha

was possible.

The harmonized hybrid framework comprised seven main steps. These are introduced in brief below, along with the context they are used for the MCs. The fulfillment of the seven steps for MC read-across is described in Section 3. The steps applied were:

i) Decision Context. This is a general consideration of the read-across assessment required. For this study, this related to the health context of the MCs and to the overall concern of the number of congeners.
ii) Data Gap Analysis. The number and types of data available for MCs were reviewed and established. This included consideration of liver tumor formation and other factors derived from mechanistic understanding. Patlewicz et al. (2018) recommend consideration of a relevant Adverse Outcome Pathway (AOP), which could lead to the use of an Integrated Approach to Testing and Assessment (IATA). However, it is noted that there is no AOP or IATA appropriate for the MCs. Patlewicz et al. (2018) also recommend the use of quantitative structure-activity relationships (QSARs), where appropriate. Again, this was not possible for the MCs.
iii) Overarching Similarity Rationale. For the MCs, this was a consideration of the structural similarity of the congeners, to support the read-across hypothesis. This step also allows the relevant RAAF scenario to be identified, which will support the similarity consideration as well as the assessment of uncertainties.
iv) Analogue Identification. This step identifies the MC analogues. In essence, potential analogues are clearly defined as being MC congeners. Therefore, there is no need to search systematically for analogues in this investigation.
v) Analogue Evaluation. The analogues identified in Step iv) were evaluated. To achieve this, a variety of data – experimental and *in silico* – were compiled as part of the supporting data matrix. This covered relevant physico-chemical properties, predictions of toxicity-related characteristics (e.g. *in silico* screens) and experimental data.
vi) Data Gap Filling. The possibility of filling data gaps for the tumor-promotion capabilities of MCs was considered within the context of the relevant RAAF scenario.
vii) Uncertainty Assessment. The ECHA RAAF was used as the basis to determine and (semi-)quantify the uncertainties associated with the read-across.

3. Results: read-across of the tumor promotion of MC congeners

The read-across of the tumor promotion of the MCs was organized based on an adapted form of the harmonized hybrid framework proposed by Patlewicz et al. (2018) (summarized in Section 2). The analysis in the seven steps of the framework is described below.

3.1. Decision context and initial uncertainties

MCs are commonly found in cyanobacterial blooms and are considered globally as one of the most hazardously known chemical groups (Bouaïcha et al., 2019). Despite the significant amount of available information on MC-LR, the most common and most toxic congener, interest in MCs continues to increase due to their well-known hazards towards human health, especially related to long-term exposure and potential carcinogenicity (WHO, 2020). A comprehensive database of cyanobacterial secondary metabolites, termed CyanoMetDB, enumerated 310 presently-known MC variants (Jones et al., 2021). Although an increasing number of MC forms are being identified, most of them are not available in sufficient purity and/or quantity for the purposes of *in vitro* or *in vivo* testing. Therefore, the adoption of the use of *in silico* predictions could, in a manner which is both rapid and cost-efficient, assist with the filling of toxicological data gaps relating to the uncharacterized congeners (Altaner et al., 2020). In addition, *in silico* methods may increase the understanding of MC toxicity, and further aid in prioritization of the large number of congeners for subsequent experimental testing.

Considering this scenario, the present read-across process is intended to fill data gaps for the human health effects of MCs by applying a generic framework to organize and capture knowledge and data relating to the substance class. Due to their molecular size and variation in component amino acids and functional groups, MCs are a structurally complex group of compounds. This makes the traditional use of computational approaches common to *in silico* toxicology more challenging (as summarized in Section 1.1). The challenges can be identified and provide a provisional starting point to understand the uncertainties inherent with read-across approaches for large, complex molecules. To respond to such challenges, and assist in the reduction of uncertainties, the use of NAMs presents a promising approach towards this (Daston et al., 2022; Marx-Stoelting et al., 2023) since it relies on tiered combinations of *in vitro* systems and advanced tools, such as omics technologies and/or *in silico* tools, to facilitate robust safety decision-making without animal testing. NAMs can enhance the translation of chemical effects into human health outcomes based on a mechanism-driven approach (Schmeisser et al., 2023). In particular, omics technologies (e.g., transcriptomics, proteomics, and metabolomics) enable the interrogation of a broad range of biomolecules in a targeted or untargeted manner, allowing for comprehensive and quantitative evaluation of biological responses at the molecular level (Viant et al., 2024; Barnett et al., 2025). Additionally, the integration of multi-omics data is seen as key to achieving comprehensive molecular response coverage, alongside a holistic biological understanding of how chemical exposure impacts human health (Brockmeier et al., 2017). NAMs require small amounts of test compound to generate plentiful data and could facilitate the understanding of the tumor promotion effect of, at least, the most relevant MC congeners. However, careful selection of appropriate *in vitro* models (e.g., hepatocyte models that adequately express organic anion transporting polypeptide (OATPs)) (Fischer et al., 2010; Le Vee et al., 2013; Badée et al., 2015; Ikehara et al., 2015) and exposure durations to account for the time-dependent biological effects of MCs (Sun et al., 2011; WHO, 2020; Ikumawoyi et al., 2024; Buratti et al., 2017) is crucial to ensure that the generated data accurately reflect the molecular mechanisms underlying the desired biological response, specifically the tumor promotion activity. It is acknowledged that the approach employed would be restricted if intended to support a regulatory dossier. The level of uncertainty for hazard identification should be commensurate with that proposed by the relevant ECHA RAAF scenario for hazard screening and the RAAF read-across scenario identified.

3.2. Data gap analysis

Based on studies with MC-LR, there is a generally accepted sequence of cellular/molecular processes, or mode of action (MOA), resulting in MC toxicity, which is the basis of the functional or physiological changes following exposure. The inhibition of protein phosphatase 2A (PP2A), leading to downstream protein hyperphosphorylation and thus reversing the action of protein kinases which regulate a diverse set of cellular processes (e.g., apoptosis, metabolism, proliferation, DNA repair), is considered the key primary event of MC-induced toxicity. This initiates alterations to cell function, followed by apoptosis and necrosis. In the liver, relatively high doses of MC lead to acute loss of cell morphology and cell-to-cell adhesion. Current studies indicate that MC exposure is not only associated with the development of liver cancer, but also non-alcoholic fatty liver disease and liver fibrosis (Zhao et al., 2020). At lower doses, typical of repeated long-term exposure, phosphatase inhibition induces cellular proliferation, hepatic hypertrophy and tumor promotion activity. In particular, MCs are potent inhibitors of the catalytic subunits of ser/thr-PPs. Their cellular uptake is primarily

dependent upon the type and level of OATP expression, as well as the capacity shown by these in transporting different congeners (Altaner et al., 2019).

The association of MC-LR with tumor promotion in the liver is well-studied. There are a number of historical studies that confirm liver tumor promotion for MC-LR. Nishiwaki-Matsushima et al. (1992) reported the outcomes of two intraperitoneal medium-term liver experiments in rats, in which tumor promotion activity was demonstrated. In the first, groups of male rats received diethylnitrosamine (DEN) a well-known tumor initiator, followed by injections of pure MC-LR, twice a week. This was followed by partial hepatectomy at the end of week three and by animal sacrifice at the end of week eight. In the second experiment, groups of rats received initiator and intraperitoneal injections of pure MC-LR, followed by partial hepatectomy in the third week. Doses of MC-LR were subsequently administered twice a week, before animals were sacrificed at the end of week eight. Tumor promotion activity was estimated in both experiments by induction of glutathione S-transferase, placental form (GST-P) foci in rat liver. This conclusion was confirmed courtesy of a repeated dose MC-LR intraperitoneal administration using either aflatoxin B1 (AFB1) or DEN as initiators, both of which are regarded as indicators for potential tumor formation (Sekijima et al., 1999). Although neither were guideline studies, nor conducted under Good Laboratory Practice (GLP) conditions, they are of sufficiently high quality for IARC to base its classification of MC-LR as possibly carcinogenic to humans (Group 2B).

Several other *in vivo* and *in vitro* studies using MC-LR were conducted. Žegura et al. (2003), using MC-LR treated HepG2 cells, demonstrated the increased activity of oxidative DNA damage-specific enzymes, thus providing evidence that MC-LR can induce DNA strand breaks in liver-derived cells. Bouaïcha et al. (2019) showed that MC-LR can induce accumulation of reactive oxygen species (ROS) in hepatocytes, this being a recognized liver cancer-initiating stress response, thereby contributing to the formation of hepatocellular carcinoma (HCC). Sun et al. (2011) discovered mRNA-induced accumulation of ROS in mouse liver acutely treated with MC-LR at 40 g/kg^{-1}. Liu et al. (2016) reported MC-LR to promote cell proliferation in the livers of mice (*in vivo*) by the activation of Akt and MAPK signaling pathways due to PP2A inhibition. They also showed, in a previous study, proliferative effects of MC-LR in HL7702 cells (immortalized human liver cell line) with the involvement of PP2A inhibition, causing the molecular changes underlying this effect. Ma et al. (2018) showed experimentally that chronic exposure for 83 days to MC-LR can increase the level of ROS in HepG2 cells, reinforcing the significance of their role in the tumor-promoting mechanisms of MC-LR, and the involvement of oxidative DNA damage in the process. Xu et al. (2018) and Zhao et al. (2012) have demonstrated, using mouse liver cancer models, the deregulation of miRNAs involved in several pathways related to tumorigenesis after exposure to MC-LR. The involvement of the epigenetic regulation by miRNAs, as well as alterations in DNA methylation, in the MC-LR-induced hepatocarcinogenesis was confirmed by Chen et al. (2018) and Zhao et al. (2019), using the human hepatocyte L02 cell line. Gu et al. (2022) demonstrated that exposure to MC-LR at 15 $\mu g/kg^{-1}$ could induce liver fibrosis in mice, an effect related to HCC. More recently, Ikumawoyi et al. (2024), using human differentiated HepaRG cells, demonstrated changes in global protein phosphorylation and dose-dependent MC-LR covalent binding towards proteins. They suggested that MCLR-mediated changes in protein phosphorylation involve biological pathways related to carcinogenesis that may contribute to the development of HCC. The *in vitro* activation of NF-κB, the phosphatidylinositol 3-kinase (PI3-K)/AKT–mediated MMP-2/9 hyperexpression observed *in vivo* and the modulation of tumor necrosis factor alpha (TNF-α) support the tumor-promoting activity of MCs through PP2A inhibition. Current studies indicate that MC exposure is not only associated with the development of liver cancer, but also non-alcoholic fatty liver disease and liver fibrosis (Zhao et al., 2020).

In addition, the tumor suppressor role of PP2A, and its activity in regulating mitogenic signaling pathways in cancer pathogenesis in mammalian cells, are both well-studied (Buratti et al., 2017). PP2A has been extensively observed to be deregulated in several tumors of cancer patients. Okadaic acid (OA), a PP2A inhibitor, was applied onto the skin of mice and lead to subsequent development of tumors, indicating that a loss of PP2A activity can assist in cancer development (Johnson et al., 2024). Several investigations have revealed a positive correlation between the expression levels of PP2A subunits and cancer aggressiveness, and further studies have elucidated this association to be driven by pathways such as PI3K-AKT, Wnt, mTOR, and MAPK (Johnson et al., 2024; Qi et al., 2024; Yu et al., 2018). Recent studies have firmly established that PP2A acts as a tumor suppressor, although underlying mechanisms are complex and not totally understood (Qi et al., 2024). At lower doses, typical of repeated long-term exposure, phosphatase inhibition induces cellular proliferation, hepatic hypertrophy and tumor promotion activity (WHO, 2020).

As the association of MC-LR with the tumor promotion in the liver is well-established and ultimately dependent upon molecular structure, it was assumed that this endpoint could be read across to fill data gaps with supporting evidence from further *in silico* and *in vitro* studies. While a common MOA is accepted for MCs as a general class, very few data are available for the remaining congeners. Accordingly, this justifies the search for a protocol enabling the use of computational methods for the purposes of filling data gaps.

3.3. Overarching similarity rationale

The initial similarity hypothesis in the read-across was based around structural similarity. Namely, all MCs congeners form a class of monocyclic heptapeptides, composed of both standard and unconventional amino acids (see generic structure of MC-LR, presented in Fig. 1). These comprise two variable L-amino acids, three common D-amino acids (or their derivatives), and two novel D-amino acids. Although variability may occur at any of the seven positions in the chain, it is most frequently observed at positions two and four. This is a fact which is reflected within class naming conventions: MC-*XY*, where cyclo-(DAla1-L-*X2*-D-isoAsp3-L-*Y4*-Adda5-D-isoGlu6-Mdha7) (WHO, 2020). All MCs contain in common the functional moieties Mdha (N-methyldehydroalanine) and Adda (β-amino acid), each of which is considered to be involved in liver toxicity as a PP inhibitor (Altaner et al., 2019; Bouaïcha et al., 2019). Variations at positions 2- and 4- are suggested to affect significantly the acute toxicity (WHO, 2020). Congeners containing a hydrophobic L-amino acid at these points, such as MC-LR, MC-LA, MC-YR, MC-YA have similar LD50 values, whereas the LD50 of the more hydrophilic MC-RR is approximately ten times higher (Xu et al., 2022). *In vivo* toxicity differences among the MC congeners can be attributed to differential uptake into the liver (via OATPs), and/or the inhibitory

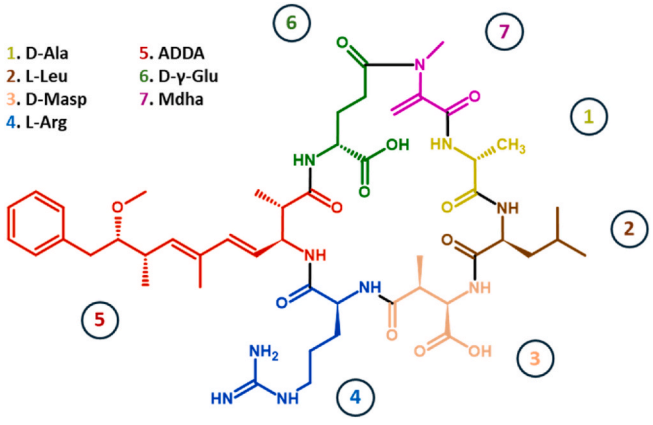

Fig. 1. Microcystin-leucine arginine (MC-LR), the most common congeners employed as the source molecule.

potency toward those hepatic PPs, which itself is widely recognized as the principal molecular initiating event (MIE) target of the class (Bouaïcha et al., 2019).

ECHA's RAAF (ECHA, 2017) was employed as a guide to analyze the read-across similarity hypothesis, similarities and uncertainties in order to assess whether the proposed reading across would be acceptable, considering definable uncertainties. The RAAF includes different approaches, described as "scenarios", containing several assessment elements (AE) which reflect a critical scientific aspect and questions of the read-across and guide its reliability evaluation. RAAF's scenario 4 was chosen, since quantitative variations in the effect caused by exposure to the source and target substances via a common mechanism was expected.

3.4. Analogue identification

Seventeen MCs were selected as the analogues as they share common structural characteristics and a common MOA. PP2A inhibition, demonstrated by experimental *in vitro* data (IC50), is reported to be the key primary event of MC-induced toxicity, leading further to several cellular events (e.g., apoptosis, proliferation, DNA repair). Similar toxicity was observed for congeners containing a hydrophobic L-amino acid in positions 2 or 4: MC-LA and MC-YR. The more hydrophilic MC-RR presented a LD50 ten times higher.

For the determination of PP2A activity, the selected MCs were tested by different methods, thus bringing variability of results and limitations for data comparison. Colorimetric assays, such as malachite green and p-Nitrophenyl Phosphate (pNPP) and fluorogenic assays, such as 6,8-difluoro-4-methylumbiliferyl phosphate (DiFMUP) and 4-methylumbelliferyl phosphate (MUP), besides the radioactive release and electrochemical biosensor assays, were employed. Different origins of PP2A present in the assays were reported: human (hepatocytes, red blood cells, recombinant PP2A catalytic subunit), bovine (heart, kidney), fish (liver), mouse (brain, skin) and rabbit (skeletal muscle). Nevertheless, values from the recombinant human PP2A catalytic subunit assay (colorimetric, pNPP) were available for most congeners (13/18), varying from 0.032 nM to 0.290 nM. The lowest and highest reported IC50s obtained from different assays are summarized in Table 2. The complete list of assays with respective origin, method and values are provided as Supplementary Information (Table S1).

Intraperitoneal (ip) LD50 values, obtained in several studies on mice, ranged from 25 to 150 μg/kg for different MCs. Similar toxicity was observed for congeners containing a hydrophobic L-amino acid in positions 2 or 4, such as the source compound MC-LR, while the more hydrophilic MC-RR presented a LD50 ten times higher (Table 2).

Structural similarity is at the heart of read-across, and this formed the first step in the analysis. Similarity between MC-LR and other MC congeners was expressed using Tanimoto coefficient (Tc) calculated from PubChem fingerprints, as retrieved from the OECD QSAR Toolbox (v.4.7). It is well recognized that the overall similarity value is dependent upon the method applied, i.e., the metric (here the Tanimoto coefficient) and the basis of structural expression (i.e., fingerprint series (Mellor et al., 2019). Similarity relative to MC-LR was extremely high, standing at 100 % for three congeners (including MC-RR) and at around 90 % (with a range of 83.4 %–99.3 %) for most others. MC-WR was least similar (at 72.1 %) (Table 3). An examination of the similarity scores calculated for a subset of the MC congeners using four different established fingerprinting algorithms supported the choice of the PubChem fingerprint. A similar ranking/clustering was obtained with the alternative fingerprints, but the PubChem fingerprint provided the best differentiation potential with a span of values from 72 to 100 % (Table S2).

Physico-chemical properties drawn upon included molecular weight (MW), logarithm of the octanol-water partition coefficient (log P), logarithm of water solubility (log WS), logarithm of vapor pressure (log VP), boiling point (BP) and melting point (MP) are reported in Table 4. Physico-chemical properties were retrieved by Search from the National Toxicology Program's Integrated Chemical Environment version 3.7 (ICE3.7), released October 2022 (https://ice.ntp.niehs.nih.gov/) on 19 August 2025. Specifically, data were retrieved from the "OPERA-predicted Physicochemical Properties" dataset, which were computed using OPERA (ver 2.8) (Mansouri et al., 2018).

MCs have high MWs (varying from 967 to 1073 Da). Log WS was also high (−0.85 to −2.12 in mol/L) and VP very low. BP and MP were very similar across all (BP 318 °C to 375 °C and MP 154 °C to 180 °C). MCs are described as extremely heat stable, non-volatile and also highly soluble in water, varying at different pHs (WHO, 2020). Predicted log P values varied from 1.63 to 4.90. Nevertheless, MCs tested using OECD guideline 117 method (OECD, 2022), by (Santori et al., 2020) are described as hydrophilic (partition coefficients 1.70, 2.25 and 2.54 at pH = 7), resulting in the following ranking from most to least polar: MC-RR, MC-YR, MC-LR. Generally, most physico-chemical properties were similar among the series, except for a variation in the calculated log P values. This introduces uncertainty into the read-across, in addition to the concern over applicability domains (as noted below). Considering the high MW of the substances, such differences should be analyzed carefully. The reality is that should the uncertainty be considered too high, the only solution would be to measure the physico-chemical properties with the acceptance of the associated time and cost.

Several *in silico* tools, both free and commercially available, were employed to provide evidence of the similarities and differences between the target and source substances. These included models and profilers related to hepatotoxicity, genotoxic and non-genotoxic carcinogenic effects, as presented in Table 5.

Hepatotoxicity: Results from computational profilers relevant to the investigated hypothesis confirmed the presence of alerts for hepatotoxicity in all MCs (moderate reliability) using the VEGA IRFMN model, and with high probability using ADMETlab2.0 (H-HT: human hepatotoxicity >0.9). However, Derek Nexus (version 6.3.0 by Lhasa Limited) fired hepatotoxicity alerts only for MC-YR, MC-LY, and MC-HtyR (Table S3).

Genotoxicity in silico: Alerts for point mutation were not identified, except for within the OECD QSAR Toolbox OASIS DNA binding profiler (which detected one genotoxic alert corresponding to Michael addition), and the *in vitro* mutagenicity tool (from Toxtree), which detected a corresponding genotoxic alert (α, β-unsaturated carbonyls) across all MCs (Table S4). It needs to be noted that the reliability of the predictions for the complex MC congener structures was low and led to variable results of overall genotoxic predictions. For example, unclassified features were reported for MC-LR by Derek Nexus, indicating that essential parts of the molecules are not represented in the validation database. Nevertheless, Derek Nexus chromosome aberration (CA) *in vitro* profiler fired an alert only for MC-YR and MC-HtyR congeners, while the Leadscope CA *in vitro* model predicted MC-LR and most others (except for MC-LA and MC-LY) as positive. The Leadscope *in vitro* SCE (sister chromatid exchange) model was negative for MC-HtyR variants and for others (MC-YR, MC-LY and MC-WR). The *in vivo* micronucleus IRFMN algorithm based on KNN and Sarpy models from VEGA platform also showed variable results, but predicted overall active/genotoxic results with moderate reliability (Table S5).

Carcinogenicity: VEGA IRFMN-ISSCAN-CGX model predicted all MCs to be carcinogenic and, although unable to perform applicability domain check, the model assigned medium to good reliability. The similarity of the analogues was high, at around 0.8. IRFMN Antares predicted the majority of MCs to be carcinogenic with lower reliability (Applicability Domain Index (ADI) = 0.5), with a few exceptions instead predicted as possibly non carcinogenic (ADI = 0.6). ISS predictions were at the limit of reliability (between 0.6 and 0.7), detecting one genotoxic alert for carcinogenicity (α, β-unsaturated carbonyls) and one non-genotoxic alert (substituted n-alkylcarboxylic acids courtesy of the ISS carcinogenicity model). On the other hand, no alerts were fired using Derek Nexus or Leadscope, in agreement with the low probability of carcinogenicity

Table 2
Microcystin (MC) mouse intraperitoneal (ip) LD50 (μg/kg bw) and IC50 PP2A (nM) (lowest and highest values) alongside corresponding origin of assay. MCs are identified by the CAS number (where available) and the DSSTox substance ID (DTXSID) as used in the US EPA CompTox Dashboard (https://comptox.epa.gov/dashboard/).

MC.CAS number and DTXSID identifier	LD50 ip [μg/kg bw]	PP2A IC50 [nM] (lowest)	Origin of lowest PP2A	PP2A IC50 [nM] (highest)	Origin of highest PP2A
MC-LR (101043-37-2; DTXSID3031654)	50	0.032	Human, recombinant PP2A catalytic subunit	7.6	Mouse skin
MC-RR (111755-37-4; DTXSID40880085)	500–800	0.056	Human, recombinant PP2A catalytic subunit	175	Human red blood cells
MC-LA (96180-79-9; DTXSID3031656)	50	0.05	Rabbit muscle	0.7	Human red blood cells
MC-YR (101064-48-6; DTXSID00880086)	70	0.09	Bovine kidney	4.5	Mouse skin
MC-LY (123304-10-9; DTXSID60891476)	90	0.34	Human hepatocyte		
MC-WR (138234-58-9; DTXSID101016178)	150–200	0.06	Human red blood cells	0.180	Human, recombinant PP2A catalytic subunit
MC-FR (No CAS; DTXSID501046711)	250	0.069	Human, recombinant PP2A catalytic subunit		
[D-Asp3,(E)-Dhb7]MC-HtyR (No CAS; DTXSID501047802)	70	0.122	Human, recombinant PP2A catalytic subunit		
[D-Asp3,(E)-Dhb7]MC-LR (No CAS; DTXSID301047549)	70	0.201	Human, recombinant PP2A catalytic subunit		
[D-Asp3,(E)-Dhb7]MC-RR (202120-08-9; DTXSID50746825)	250	2.4	Rabbit skeletal muscle	17.9–49.4	Human red blood cells
[D-Asp3,ADMAdda5,(E/Z)-Dhb7]MC-HtyR (No CAS; DTXSID701319215)	100	0.060	Bovine heart		
[D-Asp3,ADMAdda5]MC-LR (No CAS; DTXSID501047519)	160	4	Not specified		
[D-Asp3,Dha7]MC-LR (126268-88-0; DTXSID501046111)	160–300	0.254	Human, recombinant PP2A catalytic subunit		
[D-Asp3]MC-HtyR (No CAS; DTXSID701046781)	80–100	0.098	Human, recombinant PP2A catalytic subunit		
[D-Asp3]MC-LR (120011-66-7; DTXSID501016182)	200–500	0.090	Rabbit skeletal muscle		
[D-Asp3]MC-RR (118389-26-7; DTXSID201016195)	350	0.30	Human, recombinant PP2A catalytic subunit	0.45	Human red blood cells
[Dha7]MC-LR (134842-07-2; DTXSID101046169)	250	0.09	Human red blood cells	0.167	Human, recombinant PP2A catalytic subunit
[Dha7]MC-RR (131022-02-1; DTXSID101046040)	180	0.293	Human, recombinant PP2A catalytic subunit	6.6	Human red blood cells

Table 3
Structural similarity: Tanimoto coefficients (Tc) based on PubChem fingerprints to compare MC-LR and the 17 MC congeners.

MCs	Similarity to MC-LR (%)
MC-LR	100
MC-RR	100
MC-LA	95.8
MC-YR	87.7
MC-LY	83.4
MC-WR	72.1
MC-FR	96.0
[D-Asp3,(E)-Dhb7]MC-HtyR	86.5
[D-Asp3,(E)-Dhb7]MC-LR	100
[D-Asp3,(E)-Dhb7]MC-RR	100
[D-Asp3,ADMAdda5,(E/Z)-Dhb7]MC-HtyR	86.0
[D-Asp3,ADMAdda5]MC-LR	99.3
[D-Asp3,Dha7]MC-LR	99.3
[D-Asp3]MC-HtyR	87.1
[D-Asp3]MC-LR	98.6
[D-Asp3]MC-RR	98.6
[Dha7]MC-LR	99.3
[Dha7]MC-RR	99.3

(≤0.3) predicted by ADMETlab2.0 and CarcinoPred-EL (Zhang et al., 2017) as shown in (Table S6).

Acute *in vivo* data: Additional data in mice include a study conducted by Chernoff et al. (2020), in which clinical and liver-associated effects were compared among nine of the MCs herein analyzed. The study confirmed clinical changes, levels of liver enzymes, bilirubin and other liver macroscopic evaluations with different potencies for different congeners. The greatest toxicity was observed with MC-LA and MC-LR, while MC-LY and MC-YR induced similar changes to a lesser extent and MC-RR and its derived congeners showed still lower potency (Table S7).

For all *in silico* models utilized in gaining lines of evidence to support a read-across, an understanding and appreciation of the applicability domain is vital. The applicability domain for an *in silico* model is a complex concept that incorporates the physico-chemical and structural properties, as well as, if appropriate, the mechanistic and metabolic capacities of the training set (Dimitrov et al., 2005; Netzeva et al., 2005). A fundamental assumption in the use of predictions from computational toxicology is that a reliable prediction requires not only a valid model, but that the target molecule is within the applicability domain of the model (Myatt et al., 2018). For all *in silico* predictions reported in Tables 4 and 5 and the supplementary information, the placement of the MCs within the applicability domain should be evaluated. Most OPERA predictions for physico-chemical properties (reported in Table 4) are noted as being outside of the applicability domain. This is inevitable as the training data for these QSARs are likely to be based upon small molecules, typically pharmaceuticals, biocides or high tonnage chemicals. This implies that the predictions are not reliable and they should not be considered in absolute terms, i.e. the predictions of properties are not precise and should not be used outside of the context of providing weight of evidence. However, it is our assumption that the predictions are used solely to identify potential trends and large differences between the target molecule and potential analogues for read-across, as has been considered previously by Schultz et al. (2015) and Pestana et al. (2021). Further, properties can assist in the definition of applicability domains for read-across approaches themselves (Bennekou et al., 2025; Pestana et al., 2022).

Table 5
In silico profilers and platforms employed for the purposes of providing information to the target and source substances on potential hepatotoxicity, genotoxicity and carcinogenicity effects.

Profiles	Source
Bacterial Mutation Alerts	Leadscope (ver 2023)
Mutagenicity *in vitro* bacterium Alert	Derek Nexus (ver 6.3.0)
In vitro chromosomal aberration	Leadscope (ver 2023)
Chromosome damage *in vitro* mammal	Derek Nexus (ver 6.3.0)
DNA alerts for AMES, CA and MNT (OASIS)	OECD Toolbox (ver 4.7)
DNA binding (OASIS)	OECD Toolbox (ver 4.7)
Protein binding alerts for CA (OASIS)	OECD Toolbox (ver 4.7)
In vitro mutagenicity (ISS)	Toxtree (v 3.1)
Hepatotoxicity IRFMN model	VEGA (ver 1.1.3)
Assessment MN *in vivo* IRFMN model	VEGA (ver 1.1.3)
Assessment chromosome aberration	VEGA (ver 1.1.3)
Assessment MN *in vivo* Sarpy model	VEGA (ver 1.1.3)
Assessment MN *in vivo* KNN model	VEGA (ver 1.1.3)
Alerts for hepatotoxicity	Derek Nexus (ver 6.3.0)
H-HT (Human hepatotoxicity)	ADMETlab2.0
Hepatotoxicity model (IRFMN)	VEGA (ver 1.1.3)
Carcinogenicity alerts by ISS	Toxtree (v 3.1)
Carcinogenicity model (IRFMN-Antares)	VEGA (ver 1.1.3)
Carcinogenicity model (IRFMN-ISSCAN-CGX)	VEGA (ver 1.1.3)
Carcinogenicity alerts	Leadscope (ver 2023)
Carcinogenicity alerts	Derek Nexus (ver 6.3.0)
Carcinogenicity	ADMETlab2.0
CarcinoPred-EL	Zhang et al. (2017)

3.5. Analogue evaluation

A consistent data matrix according to ECHA (2017) is presented in Tables 6a and 6b (common AEs) and (specific scenario AEs) for analogue evaluation in which the characteristics effects were discussed for source and target congeners. Chemical similarity was confirmed in that all MCs

Table 4
Predicted physicochemical properties, including molecular weight (MW), logarithm of the octanol-water partition coefficient (log P), logarithm of water solubility (log WS), logarithm of vapor pressure (log VP), boiling point (BP) and melting point (MP). All values calculated from OPERA (ver 2.8) available from ICE3.7.

NAME	MW	Log P	Log WS mol/L	Log VP (mmHg)	BP (°C)	MP (°C)
MC-LR	995	3.85	−2.12	−7.60	323	154
MC-RR	1038	1.63	−1.23	−8.72	326	156
MC-LA	909	3.71	−2.00	−6.85	318	180
MC-YR	1045	4.88	−1.58	−7.59	324	167
MC-LY	1002	4.90	−1.73	−7.43	375	180
MC-WR	1068	4.19	−1.83	−7.57	343	167
MC-FR	1029	4.88	−1.4	−7.55	341	174
[D-Asp3,(E)-Dhb7]MC-HtyR	1045	4.47	−1.58	−7.60	325	168
[D-Asp3,(E)-Dhb7]MC-LR	981	3.83	−1.57	−7.61	324	167
[D-Asp3,(E)-Dhb7]MC-RR	1024	1.92	−0.84	−8.71	343	159
[D-Asp3,ADMAdda5,(E/Z)-Dhb7]MC-HtyR	1073	4.47	−1.57	−7.59	325	168
[D-Asp3,ADMAdda5]MC-LR	1009	4.09	−0.85	−7.58	341	164
[D-Asp3,Dha7]MC-LR	967	2.86	−1.57	−7.62	324	167
[D-Asp3]MC-HtyR	1045	4.88	−1.58	−7.60	325	167
[D-Asp3]MC-LR	981	3.86	−2.12	−7.61	324	157
[D-Asp3]MC-RR	1024	1.98	−1.23	−8.72	326	156
[Dha7]MC-LR	981	3.84	−1.57	−7.61	324	160
[Dha7]MC-RR	1024	1.95	−1.57	−8.72	326	156

Table 6a
Data matrix according to European Chemical Agency's (ECHA's) Read-Across Assessment Framework (RAAF) and its Assessment Elements (AEs) (ECHA, 2017).

AE	RAAF AE	Explanation	Score	Data	Major uncertainties
AE C.1	Substances characterization	Defined structures	5	PubChem	None; the source substance, as well as the group of analogues used as targets, have clear chemical identities and characterization.
AE C.2	Structural similarity and structural differences within the category	Defined category considering chemical structure; range of structural similarity.	4	MCs are cyclic peptides with a ring structure of seven variable amino acids. Similarity range using PubChem fingerprint: 72–100 %.	Although similarity was high among the substances, no 3D structure was used to compare them.
AE C.3	Structural similarities and structural differences with the proposed regular pattern	Defined category considering physicochemical properties	3	PC properties (range): MW 967 to 1073Da; log WS -0.85 to −2.12 mol/L; Log P 1.63 to 4.90.	Physicochemical properties were calculated and may not reflect experimental values, which is not typical in read-across studies. Many calculations are outside of the applicability domain of the models and should be used only to determine trends and significant differences.
AE C.4	Consistency of effects in the data matrix	Comparison of experimental data (data matrix); available data show consistent properties across the data matrix.	3	There is a consistent data matrix with same effects for all substances: LD50 and PP2A values.	Several different experimental methods were used for PP2A determination, which makes the comparison among the values more difficult.
AE C.5	Reliability and adequacy of the source studies	Quality and consistency of data	4	Three i.p. *in vivo* studies report MC-LR as a liver tumour promoter; considered fit for purpose.	Although high quality and fit for purpose, *in vivo* studies for the endpoint (tumor promotion) are not guideline studies nor suitable for regulatory purposes.
AE C.6	Bias that influences the prediction	Information that influences predictions of category members.	5	All *in vivo* studies are presented; all available data is employed in the discussion.	There are few *in vivo* studies on this specific endpoint and no bias influenced the selection of the source studies.

are cyclic peptides with a ring composed of seven variable amino acids. Structural similarity was considered high, ranging from 72 to 100 % as determined using PubChem fingerprints. Generally, most physico-chemical properties were similar among MCs.

There are experimental data on the mechanistic hypothesis for all substances (IC50 PP2A values). These show little variability in IC50 values across the MC congeners. Variability in the methods of PP2A activity determination could be minimized by selecting data from a single species and from similar detection techniques. Overall, there is a consensus that different congeners vary in their potency of inhibiting PP2A activity, although the scale of variation is relatively modest (WHO, 2020).

There are robust *in vivo* data in rodents for the source compound, which indicate a tumor promotion potential. There are, in addition, a few *in vivo* toxicity studies, within which a variety of MCs show hepatotoxicity. Although *in vivo* data for the source compound demonstrates tumor promotion, the corresponding evidence within targets is less convincing. LD50s determined in all MCs showed different potencies, with similar high toxicity for the source congener MC-LR and also for others containing a hydrophobic L-amino acid in positions 2- or 4-. The more hydrophilic MC-RR presented a LD50 ten times higher. These effects were confirmed by confirmed by the study of Chernoff et al. (2020).

It was suggested that most of the *in vivo* toxicity variability among MC congeners could be attributed to a combination of differences in their uptake into the liver and/or their inhibitory potency toward hepatic PPs. Acute *in vivo* MC toxicity depends on OATP transport kinetics, with both OATP and PP2A activities correlated to hydrophobicity. However, the relative importance of each component towards MC toxic potency remains controversial (Bouaïcha et al., 2019). Furthermore, the differences observed are not directly related to the endpoint. Overall, no *in vivo* evidence disproves the read-across hypothesis.

Table 6b
Data matrix according to RAAF and Specific Scenario AEs (ECHA, 2017).

AE	RAAF AE	Explanation	Score	Data	Major uncertainties
AE 4.1	Compounds the test organism is exposed to	Compounds cause the same effects through a common mechanism; consistency in the ***in vivo*** effects	3	There are robust *in vivo* data in rodents for the source compound, showing tumor promotion and oral *in vivo* toxicity studies with a variety of MCs showing hepatoxicity.	Although *in vivo* data for the source compound demonstrate tumor promotion, the *in vivo* evidence for the targets is less convincing and is not directly related to endpoint.
AE 4.2	Common underlying mechanism: qualitative aspects	Mechanism linking the compounds driving qualitatively similar effects is identified	3	There are experimental data on the mechanistic hypothesis for all substances (IC50 PP2A values), which is the accepted MoA of MC toxicity showing that IC50 values can vary among MC variants.	Although variability of methods in the PP2A activity determination could be minimized by selecting data from a single species and/or adopting similar detection methods, results should be compared with caution.
AE 4.3	Common underlying mechanism: quantitative aspects	Differences in kinetics and/or potency among the substances after exposure.	3	There are quantitative differences demonstrated by two lines of evidence: acute toxicity potency and transport (OATP activity).	It is demonstrated that acute i*n vivo* MC toxicity depends on OATP transport kinetics and both OATP and PP2A activities are correlated to hydrophobicity. However, these differences are not directly related to the endpoint.
AE 4.4	Exposure to other compounds/ metabolites	Other compounds may be present or formed through biotransformation pathways, as an intermediate or as impurity.	4	Information on ADME of MCs suggests that MC metabolites are less toxic than the parent molecules.	Although both *in vitro* and *in vivo* detoxification is described as conjugation with GSH and cysteine, with the liver as the major site of metabolism, few experiments were conducted.
AE 4.5	Occurrence of other effects than covered by the hypothesis	Different effects described in the toxicological profile of substances suggest other acting toxic mechanism.	3	No different effects described or obtained by the *in silico* profiles suggest other acting toxic mechanism.	Literature suggests a non-genotoxic mechanism for MCs toxicity, scarce data on most MCs and possible involvement of other pathways for the endpoint.

3.6. Data gap filling

The gaps of knowledge for the MCs could be better understood when the different lines of evidence were combined in order to form an overall weight of evidence (WoE). This analysis across studies demonstrates whether consistency is achieved and how they impact the endpoint. Two main lines of evidence were described based upon the available literature: association of PP2A with tumor promotion, and association of MC-LR with the tumor promotion MOA in the liver.

WoE related to the availability of data across the literature for the congeners is analyzed to better understand the impact to the endpoint, and the activity of PP2A in other MCs (targets) in different assays is compared. Both Fischer et al. (2010) and a review by Buratti et al. (2017) demonstrated the different affinities of several MCs to OATP1B1 and 1B3 transporters *in vitro* (primary human hepatocytes and HEK293 cells). More hydrophobic congeners showed greater effect than MC-LR. MC-RR, the more hydrophilic, was the least active towards OATP. Also, differences in detoxification efficiency have been used to explain variation in congener-specific toxicity across MCs. Detoxification both *in vitro* and *in vivo* is described as occurring through conjugation with GSH and cysteine, since MC-LR and MC-YR conjugates are less toxic than the parent (2- to 17-fold higher in terms of mouse LD50 value) and conjugation efficiency is congener-dependent: the total *in vitro* detoxication reaction favors variants with higher hydrophilicity, with MC-RR being conjugated at the highest rate (WHO, 2020).

Altaner et al. (2019) performed serine/threonine protein phosphatase assays with 18 MCs and demonstrated the extent of inhibitory effect in each: MC-RR, MC-LR and MC-YR presented similar IC50 for PP2A, while PP2A was ≥2-fold more sensitive to the more hydrophobic MC-LW and MC-LA Xu et al. (2022) measured relative potency of *in vitro* PP2A inhibition, with the assistance of molecular modeling, to be: MC-LR > MC-LW > MC-LA > MC-LY. A statistical correlation was observed between the hydrophobicity of Z4 and MC toxicity.

Altogether, WoE points out that the similarities related to MOA may be modulated by toxicokinetic and toxicodynamic aspects. The combination of several *in silico* tools was reported to address the differences in predictions which emerged between tools used in examination of mycotoxins and MCs (da Silva et al., 2021; Lemée et al., 2023). Nevertheless, such a combination could not fill the gaps described in our study, likely due to the high molecular weight of the analyzed molecules. Literature suggests a non-genotoxic mechanism for MC toxicity, with secondary genotoxicity, associated with reactive oxygen species (ROS) formation, resulting in oxidative DNA lesions. This stands in contrast to direct interactions with DNA leading to mutation. While our predictions also indicated MCs to be indirectly genotoxic, with medium to high confidence, the *in silico* results for carcinogenicity were conflicting. The absence of carcinogenic effects was predicted by four different models, but positive predictions on the edge of reliability were also obtained. Especially for this complex endpoint, more specific *in silico* tools for oxidative stress, chronic inflammation, immunosuppression and cell proliferation are not available. Nevertheless, these could be an extremely useful alternative to prioritize the large number of toxins and their metabolites which lack experimental results (Lemée et al., 2023; Keller et al., 2023).

Results from several studies indicate that most MC variants may be similar with respect to their protein phosphatase inhibiting potency, despite differences in amino acid composition. Therefore, pharmacokinetic differences among the congeners, such as differences in uptake, first-pass clearance and distribution from the gut (WHO, 2020), may be at least partially responsible for observed variations in lethality. *In vitro* kinetic data clearly indicate that MC congeners have a comparable inhibitory effect on protein phosphatases to MC-LR (Altaner et al., 2020). However, toxicokinetics may play an important role, *in vivo*, in determining the extent of the effects exerted by different variants (Santori et al., 2020).

Overall, provided the uncertainties assessed in step 7 (Section 3.7) are tolerable, the read-across of the liver tumor promotion capacity of MC congeners from MC-LR is justified.

3.7. Uncertainty assessment

Evaluation of read-across through the ECHA RAAF provided a semi-quantitative measure of confidence. The uncertainties associated with each AE were assessed using a score from 1 (i.e., not acceptable) to 5 (acceptable with high confidence). When the combination of the scientific explanation and supporting evidence is sufficient, the conclusion may be "Acceptable with high confidence, with medium confidence" (scores 5 and 4) or "Acceptable with just sufficient confidence" (score 3) as described (ECHA, 2017). As all MCs have clear chemical identities, open and unambiguous characterization, high structural similarity and like predicted physicochemical properties, then acceptable confidence in the relatedness of source and target substances could be assumed. As such, across the six common AEs (Table 6a), scores 3 to 5 were attributed. For the specific scenario (Table 6b), most AEs were judged as "Acceptable with just sufficient confidence" (scored 3), largely because, although MOA is well known, its direct relation with the endpoint is not well established. According to RAAF definitions, the read-across for tumor promotion activity in the liver, from MC-LR to other MCs, was scored as "acceptable with just sufficient confidence". Quantitative differences were demonstrated, but uncertainties associated with the quality of experimental data and gaps in mechanistic insight relating both to target and source compounds limited the overall confidence score.

Discussions on the best practices of read-across evaluation using the RAAF scheme are critical to improve its utility in regulatory submissions (Roe et al., 2025a, 2025b) and were discussed in recent publications on how to improve its use in the general regulatory context (Roe et al., 2025a, 2025b; Patlewicz et al., 2024, 2025; Schmitt et al., 2025). Structural similarity, still the most commonly used element, was confirmed as a strong predictor of read-across acceptability, together with the non-guideline toxicodynamic similarity studies, the second most common element (Roe et al., 2025a). In the present exercise, the structural similarity was confirmed to be high, and bridging studies were consistent in demonstrating a qualitatively common mode of action. Considering both quantitative *in vivo* differences, and those related to cellular uptake and detoxification efficiency, it is not unlikely that different congeners could demonstrate variable tumor promotion activity. While MC-LR activity could be considered high, MC-RR and other variants could be less effective in terms of tumor promotion potential.

Although there is general agreement as to the key processes which drive MC toxicity, no long-term carcinogenicity or chronic exposure studies are available. Also, as a significant limitation exists in the availability of high-quality, pure MC standards. Understandably, this hinders the ability to conduct animal studies to clarify the tumor promotion activity of individual, specific congeners. Also, it should be stressed that no single path to malignancy is described for non-genotoxic carcinogens. Therefore, grouping tumor promoter agents, such as phenobarbital, dichlorodiphenyltrichloroethane, polychlorinated biphenyls and others as hepatocarcinogens is a default criterion that also implies uncertainties (Stewart, 2019).

In summary, the read-across proposed in Step 6 (Section 3.6) for the MC congeners has acceptable levels of uncertainty as defined by ECHA RAAF scenario 4 for the purpose of hazard identification.

4. Discussion

This study has utilized read-across as a means to fill data gaps for the human health effects of MCs. It has applied a generic framework for read-across to organize and capture knowledge and data relating to the substance class. Generally, it was observed that MCs are data poor with regard to tumor promotion in the context of carcinogenicity. However, read-across allowed for toxicity to be inferred within the group of

congeners.

Overall, this case study demonstrates the potential applicability of *in silico* tools for regulatory applications such as prioritization for further testing or read-across and grouping for assessing large groups of complex compounds, such as MCs. Even though no single *in silico* tool on its own would, for now, deliver results that are considered valid enough to use them for *ab initio* decision making, the use of a battery of such tools increases confidence. Ultimately, for some regulations, such as the EU Contaminants Regulation that would also cover MCs, the data requirements would allow the inclusion of such data as part of the risk assessment procedure (EC, 1993). In addition, this study allowed for the identification of opportunities for the further application and development of read-across for large, complex molecules (Section 4.1) and uncertainties in such assessments (Section 4.2).

4.1. Key Successes and opportunities for using read-across for large, complex molecules

Whilst there are a number of challenges associated with the use of read-across for molecules such as the MCs, a number of strengths and positive outcomes to this analysis were identified. These include.

- The investigation revealed that read-across can be considered a viable technique to fill data gaps for structurally complex molecules of this form. As such, it has demonstrated that read-across can be applied to compounds lying outside of the usual domains of applicability.
- Whilst computational analysis of the MCs is challenging, structural alerts were found to be valuable in providing information relevant for assessing the impact of MC side chains.
- Large data matrices of supporting lines of evidence can be compiled. In isolation, these may not provide overwhelming support for a read-across justification, but combined into a WoE they can form a powerful argument.
- Rather than considering the calculated properties as definitive values, it is easier to consider the differences between these, e.g. source vs target and overall range, as part of an evaluation into the applicability domain and confidence in a specific read-across.
- Targeting of testing, e.g. with *in vitro* NAMs in key areas such as mode/mechanism of action which are essential for advancing knowledge on congener-specific toxicity and potency to enhance hazard and risk assessment.
- Integrating a number of *in silico* tools covering different endpoints into a WoE approach, combined with read-across and use of existing mechanistic information from *in vitro* NAM, can facilitate the risk assessment of a large group of toxins.
- Harmonized frameworks, such as (Patlewicz et al., 2018), whilst not perfect, do provide a means by which to direct the development of read-across hypotheses and the collection and analysis of data.
- Uncertainty assessment and RAAF are crucial to understanding the confidence that can be assigned to the read-across, and to providing a means of its justification with the intention of allowing for its acceptance for a particular purpose.
- There is a need to extend this approach and develop strong groups of structural and mechanistic analogues of molecules such as MCs.

4.2. Evaluation of uncertainties in read-across

The read-across exercise demonstrated the use of various lines of evidence to support a hypothesis of structural similarity and allowed for the consideration of uncertainties within the evidence and how it impacts a possible, overall conclusion. In this study the ECHA RAAF was utilized as a means to evaluate the read-across. This can be applied to determine uncertainties, particularly those of relevance for the regulatory application of read-across. It is notable that the RAAF provided a framework that could be applied to large complex molecules such as the MCs.

Evaluation of uncertainties in read-across is an essential process to demonstrate and justify the validity of a read-across (Bennekou et al., 2025). It is now considered vital as part of the problem formulation, and will be context, use and endpoint dependent. Going beyond the ECHA RAAF, a number of uncertainties in read-across have been identified. Schultz et al. (2019) summarized the schemes to evaluate read-across uncertainty and proposed a number of keys areas – most importantly relating to chemical similarity, toxicokinetics, toxicokinetics and data read across. These were placed in the context of existing schemes including the ECHA RAAF. Whilst uncertainties in read-across may be readily identified, their precise quantification is difficult and as yet only loosely defined in schemes such as presented by Pestana et al. (2021) and Bennekou et al. (2025). However, this study demonstrated the utility of investigation of uncertainties, for instance highly the considerable variability in log P values for MC-LR and its congeners. In this study the uncertainty was deemed acceptable, as there were several other lines of evidence that confirmed similarity. The impact of the uncertainty was, however, notable in AE C.3 (Table 6a) which lowered the overall score to 3. Should a read-across scenario be considered where this score was not deemed acceptable, then further evidence would be required to lower the score.

5. Conclusions

In conclusion, the read-across exercise for the liver tumor promotion of the MCs could be performed on the basis of a standardized framework. A variety of lines of evidence and data streams were compiled which showed consistency across the MC congeners. The ECHA RAAF provided a means to evaluate the read-across in a structured manner and to assess the associated uncertainties, which were clearly defined and discussed. The uncertainties were found to be acceptable for the purpose of data gap filling as stated in the problem formulation. We believe that the read-across strategies, with known caveats and restrictions, could be applied to other groups of large and complex molecules, including toxins.

CRediT authorship contribution statement

Cynthia B. Pestana: Writing – original draft, Conceptualization. **Daniela Morais Leme:** Writing – review & editing. **Enzo Zini Moreira Silva:** Writing – review & editing. **Sahra Kiessig:** Writing – review & editing. **James W. Firman:** Writing – review & editing. **Carsten Kneuer:** Writing – review & editing. **Philip Marx-Stoelting:** Writing – review & editing. **Mark T.D. Cronin:** Writing – review & editing, Supervision.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yrtph.2025.105938.

Data availability

Data will be made available on request.

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