Comparative effects of nodularin and microcystin-LR in zebrafish:

2. Uptake and molecular effects in eleuthero-embryos and adult liver with focus on endoplasmic reticulum stress.

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Running title: ER-stress induction of MC-LR and nodularin in zebrafish

Abstract

Microcystin (MC) and nodularin are structurally similar cyanobacterial toxins that inhibit protein phosphatases. Additional modes of action are poorly known, in particular for nodularin. In our associated work, we showed that active cellular uptake is mediated by the organic anion transporting polypeptide *drOatp1d1* in zebrafish (Faltermann et al., 2015a). Here, we assessed the transcriptional expression of three genes encoding three uptake transporters during embryonic development from 24 hours post fertilization (hpf) to 168 hpf. Transcripts of *drOatp1d1* and *drOatp2b1* are present at 24 hpf. The abundance increased after hatching and remained about constant up to 168 hpf. Transcripts of *drOatp2b1* were most abundant, while *drOatp1f* transcripts showed very low relative abundance compared to *drOatp1d1* and *drOatp2b1*. We further demonstrated the uptake of fluorescent labeled MC-LR in eleuthero-embryos and its accumulation in the glomerulus of the pronephros.

An important molecular effect of MC-LR in human liver cells is induction of endoplasmic reticulum (ER)-stress. Here, we investigated, whether MC-LR and nodularin similarly lead to induction of ER-stress in zebrafish by analyzing changes of mRNA levels of genes indicative of ER-stress. In zebrafish liver organ cultures short-term exposures to 0.5 and 1 μ M MC-LR and nodularin, respectively, led to significant transcriptional induction of several ER-stress marker genes, including the chaperone *glucose regulated protein 78 (bip)*, the spliced form of *x-box binding protein (xbps-1s)*, the *CCAAT-enhancer-binding protein homologous protein (chop)* and *activating transcription factor 4 (atf4)*. Furthermore, strong transcriptional changes occurred for *tumor necrosis factor alpha (tnfa)* and *dual specificity phosphatase 5 (dusp5)*, associated with mitogen activated protein kinase (MAPK) pathway. However, no alterations in transcript levels of pro-apoptotic genes *Bcl-2 like protein 4 (bax)* and *p53* occurred. In contrast to adult liver, MC-LR and nodularin did not result in detectable changes of mRNA levels of selected target genes involved in ER-stress in zebrafish eleuthero-embryos, nor were transcripts belonging to the MAPK and pro-apoptosis pathways altered.

In conclusion, our data indicate that MC-LR and nodularin have similar effects on transcription. They lead to changes on mRNA level of genes that suggest induction of ER-stress, and furthermore, lead to increased level of *tnfa* mRNA in the adult liver, which suggests a novel transcriptional mode of action

demonstrated in fish. However, although taken up by eleuthero-embryos, no transcriptional changes induced by these cyanobacterial toxins were detected. This is probably due to action to specific organs such as liver and kidneys that could not be identified by whole-embryo sampling.

Key Words: Cyanobacterial toxin; microcystin; nodularin; endoplasmic reticulum stress, zebrafish, liver organ culture

1. Introduction

Exposure of humans and animals to cyanobacterial toxins from cyanobacterial blooms is an increasing global problem due to intensifying eutrophication and global climate change (Paerl and Paul, 2012). In freshwater, microcystin (MC) producing cyanobacteria are of importance, whereas in brackish water, nodularin producing cyanobacteria are relevant. Cyanobacterial blooms may lead to toxicity of fish populations (Wiegand and Pflugmacher, 2005). Whereas the effects of MCs are fairly well investigated, effects of nodularin are less known. However, these cyclic heptapeptides share structural similarities, and toxicokinetic and toxicodynamic properties (Fujiki and Suganuma, 2011). Due to their large size, cellular uptake functions by active transport via organic anion transporting polypeptides (Oatp). In zebrafish liver, *Oatp2b1* and *Oatp1d1* are predominant (Popovic et al., 2010). In our associated paper, we showed that *Oatp1d1* mediates cellular uptake of MC-LR and nodularin (Faltermann et al., 2015a). In contrast to cellular expression, at present nothing is known about the expression of these uptake transporters in zebrafish embryos and eleuthero-embryos. Early embryos are protected from MC-LR (Oberemm et al., 1997), as long as they are not hatched, because the chorion probably acts as barrier for the cyanotoxin (Wang et al., 2005). Similar to MC-LR, nodularin accumulates in the liver. In the liver of European flounders, nodularin concentrations of up to 557 $\mu\text{g kg}^{-1}$ have been detected (Persson et al., 2009).

The main target organ of MC-LR, and potentially nodularin, is the liver (Svircev et al., 2010), which expresses high levels of many uptake transporters, including Oatps (Roth et al., 2012). One of the important molecular toxicological mechanism of these toxins is the inhibition of serine/threonine specific protein phosphatases PP1 and PP2A (MacKintosh et al., 1990). This in turn leads to hyperphosphorylation of proteins, ultimately resulting in deterioration of cellular integrity.

Thus far, little is known on additional molecular effects of nodularin. For MC-LR our group demonstrated an induction of endoplasmic reticulum (ER)-stress and associated unfolded protein response (UPR) in a human hepatoma cell line (Christen et al., 2013). The ER has various important functions, including protein folding. Disturbance of protein structure and functions, either induced physiologically or toxicologically, results in accumulation of unfolded or misfolded proteins and leads to ER-stress (Malhotra and Kaufman, 2007). This molecular mechanism is involved in the

pathogenesis of many diseases, including neurological (Paschen and Mengesdorf, 2005) or metabolic disorders (Ozcan and Tabas, 2012). During prolonged ER-stress, UPR is activated to restore the normal function of the ER and stabilize protein folding. The UPR is highly conserved in animals and acts via three different pathways resulting in decreased protein translation, increased protein degradation and up-regulation of molecular chaperones, like the glucose regulated protein 78, also called bip (Malhotra and Kaufman, 2007). Transcriptional induction of *bip*, as well as splicing of the *x-box binding protein 1* (*xbp-1*) are key processes in the UPR and can be used for ER-stress determination (Arends et al., 2013). However, changes in mRNA levels need not consequently result in changes on the protein level. Nevertheless, also up-regulation of *chop* is an indicator of ER-stress and its protein is mainly responsible for apoptosis, which is induced, when normal functions are not regained (Samali et al., 2010).

Oxidative stress is closely linked to ER-stress (Malhotra and Kaufman, 2007), which can result in apoptosis. Oxidative stress is reduced by several enzymes, including catalase, superoxide dismutase and glutathione peroxidase (Malhotra and Kaufman, 2007). Also, the phase II enzyme glutathione-S-transferase (GST) protects cells from oxidative stress and reactive oxygen species (Hayes and Pulford, 1995). Activation of oxidative stress by MC-LR and nodularin was shown in aquatic animals (Amado and Monserrat, 2010). Another pathway that is affected by MC-LR is the mitogen-activated protein kinases (MAPK) signaling pathway. Interaction of MC-LR and the MAPK signaling cascade occurs via induction of tumor necrosis factor alpha (TNF α), an endogenous tumor promotor (Fujiki and Suganuma, 2011; Christen et al., 2013). After binding to its receptor, TNF α can induce all three MAPK signaling pathways (p38, ERK1/2 and JNK) resulting in the regulation of numerous cell functions including proliferation, apoptosis and differentiation (Marques-Fernandez et al., 2013; Wajant et al., 2003).

The aim of our present work was to investigate the expression of *Oatps* during early life stage development and to determine the uptake of MC-LR into zebrafish by use of a newly synthesized fluorescent derivative of MC-LR (Grundler et al., 2015). Furthermore, we aimed to investigate the ER-stress induction, as well as the oxidative stress response in liver organ cultures of adult zebrafish and in eleuthero-embryos exposed to MC-LR and nodularin. Our data are based on changes in mRNA

levels (and not protein levels), but nevertheless suggest that MC-LR and nodularin act similarly by induction of ER-stress, representing a novel transcriptional mode of action in fish.

2. Material and Methods

2.1 Chemicals and reagents

Microcystin-LR (MC-LR) and nodularin were purchased from Enzo life-science (Lausen, Switzerland). Reconstituted fish water was prepared as followed: Deionized water with ions added ($\text{CaCl}_2 \times 2\text{H}_2\text{O}$ 147.0 mg L⁻¹, KCl 2.9 mg L⁻¹, $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ 61.6 mg L⁻¹, NaHCO_3 32.4 mg L⁻¹) and a conductivity of 470 – 480 µS/cm. Texas red was purchased in form of succinimidyl ester from LuBioScience (Luzern, Switzerland).

2.2 Animals

Fertilized eggs and adult zebrafish were obtained from Harlan Laboratories Ltd (Itingen, Switzerland). Adult zebrafish were maintained in the laboratory as previously described (Blüthgen et al., 2013).

2.3 Exposure of zebrafish early life stages to fluorescent MC-LR, cryo-sectioning and imaging

Exposure of zebrafish eleuthero-embryos started after hatching at 72 hpf in 48-well plates (Huberlab, Reinach, Switzerland), containing 500 µL reconstituted fish water per well, with 0.01 % DMSO (solvent control), 1 µmol L⁻¹ MC-LR-Texas red (Grundler et al., 2015) or 1 µmol L⁻¹ Texas red (negative control). In addition 1 µmol L⁻¹ MC-LR labeled with Alexa 488 (Grundler et al., 2015) has been employed for analysis of uptake into early embryos. The first fluorophor that we coupled to MC-LR was Alexa 488, therefore we did our first experiments with MC-alexa488. Afterwards, we also tested other fluorophores, including Texas red, that was previously used for labeling gentamycin for uptake experiments with zebrafish eleuthero-embryos (Wang and Steyger, 2009). MC-LR-Texas red was best concerning uptake by drOatp1d1, signal intensity and conditions for imaging of zebrafish eleuthero-embryos in respect to autofluorescence.

After exposure for 96 h, 168 hours post fertilization (hpf) eleuthero-embryos were fixed in 4 % paraformaldehyde (Fluka, Buchs, Switzerland) for 4 h at room temperature, embedded in Tissue TEK (Hartenstein, Würzburg, Germany) and stored at -80 °C until sectioning. Fifty µm sections were generated with a cryostat ([HM560 MV, Thermo Scientific](#)) and analysed. Images were taken by an Olympus Laser scanning microscope FV100 (excitation: 561 nm).

2.4 Exposure of zebrafish liver organ culture for gene expression analysis

Nine months old female zebrafish were euthanized in Koi Med sleep (Schönbach Apotheke, Asslar, Germany). Subsequently, the liver was dissected and cut into four equal pieces. Equal liver pieces were randomly distributed to four dose groups of MC-LR and nodularin, respectively, consisting of a solvent control (0.01 % DMSO in HBSS buffer (LuBioScience, Luzern, Switzerland)), a low MC-LR and nodularin concentration (0.5 $\mu\text{mol L}^{-1}$ MC-LR or nodularin in HBSS buffer with 0.01 % DMSO), a high MC-LR and nodularin concentration (1 $\mu\text{mol L}^{-1}$ MC-LR or nodularin in HBSS buffer with 0.01 % DMSO), and a positive control for ER-stress induction (1 $\mu\text{mol L}^{-1}$ thapsigargin (Enzo-lifescience, Lausen, Switzerland) in 0.01 % DMSO). Exposure was performed in 96-well plates on a shaker, containing 200 μL of exposure solution per well and at constant temperature of 28 °C. Exposure lasted for 2.5 h (short-time exposure), and 5 h (longer-term exposure). Each dose group consisted of seven replicates at both exposure times. Each experiment was repeated two times. After exposure, liver pieces were fixed in RNA Later (Qiagen, Basel, Switzerland) and stored at - 20 °C until RNA extraction.

2.5 Total RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted from zebrafish tissue (liver or zebrafish eleuthero-embryos) using RNeasy Mini Kit (Qiagen, Basel, Switzerland) according to the manufacturer`s instruction. RNA concentrations and purity were measured spectrophotometrically (NanoDrop ND-1000, Witec AG, Luzern, Switzerland) and RNA integrity was provem with agarose (Axon Lab, Baden-Dättwil, Switzerland) gel electrophoresis (Bio Rad, Cressier, Switzerland). Single strand cDNA synthesis and RT-qPCR were carried out as previously described (Faltermann et al., 2014). Primers are listed in Table 1.

Quantification of *drOatp1d1* transcripts was performed according to Simon (2003), using the relative quantification method:

$$\text{MNE} = E_{\text{ref}} \wedge C_{\text{ref}; \text{mean}} / E_{\text{target}} \wedge C_{\text{target}; \text{mean}}$$

Expression was normalized to the housekeeping gene *RPL13a* expression. Expression of this gene was constant over the exposure period. For data analysis, each liver was analysed separately and transcriptional changes after exposure to MC-LR and nodularin, respectively, were normalized to the respective control piece.

2.6 *drOatp1d1* expression in zebrafish early life stages

After fertilization, embryos were raised in Petri dishes (30 mL reconstituted fish water / 15 embryos in an incubator (KBWF 240, Binder GmbH, Tuttlingen, Germany) with constant temperature (27 °C), humidity (70 %) and light dark cycle (16 h light and 8 h dark). Three replicates of 15 embryos and eleuthero-embryos, respectively, were taken from the Petri dishes and fixed in RNA Later daily from 24 hpf - 168 hpf. Furthermore, replicates of zebrafish maintained in 0.01 % DMSO in reconstituted fish water from 72 hpf to 96, 120, 144, 168 hpf were included. They were also fixed in RNA Later (three replicates per time point).

2.7 Exposure of zebrafish early life stages for gene expression analysis

After quality control under the stereo microscope (StemiD4, Zeiss.), early embryos (2-5 hours post fertilization, hpf) were transferred to 150 mL glass beakers (80 embryos per beaker) containing 100 mL of freshly prepared reconstituted fish water. Beakers were covered with lids from Petri dishes and held in an incubator with the same settings as described in 2.6. Included was a short-term and long-term exposure.

Short-term exposures were performed at 120 hpf and 168 hpf for 4 h. Thereby, 60 hatched eleuthero-embryos from each beaker were transferred into a new autoclaved beaker, containing 100 mL of freshly prepared reconstituted fish water, or 34 hatched eleuthero-embryos from each beaker were transferred to 48 well plates (one embryo per well) containing 500 µL of reconstituted fish water. At 120 hpf and 168 hpf fish water was replaced by the exposure solutions (solvent control containing 0.01 % DMSO in reconstituted fish water, MC-LR concentrations of 1 µmol L⁻¹ and 2 µmol L⁻¹ at 120

hpf, $0.1 \mu\text{mol L}^{-1}$ and $1 \mu\text{mol L}^{-1}$ at 168 hpf and $0.1 \mu\text{mol L}^{-1}$ and $1 \mu\text{mol L}^{-1}$ nodularin at 120 hpf and 168 hpf). Each dose group consisted of three replicates.

The long-term exposure started at 72 hpf for 24 h, 48 h, or 96 h in case of MC-LR and 96 h in case of nodularin. Thereby, at 72 hpf, hatched eleuthero-embryos from each beaker were transferred into a new autoclaved beaker, containing 100 mL of freshly prepared exposure solutions, or 34 hatched eleuthero-embryos from each beaker were transferred to 48 well plates (one embryo per well) containing 500 μL of exposure solution. Each dose group (solvent control containing 0.01 % DMSO, low concentration of $0.1 \mu\text{mol L}^{-1}$ MC-LR and nodularin, and high concentration of $1 \mu\text{mol L}^{-1}$ MC-LR and nodularin) consisted of four replicates.

Details of experimental setup and MC-LR and nodularin concentrations are shown in Fig 1. Every 24 h, eleuthero-embryos were observed under the stereo-microscope for sublethal or lethal effects.

2.8 Determination of MC-LR and nodularin exposure concentration

Exposure concentrations of MC-LR and nodularin were determined for the 96 h exposure of zebrafish eleuthero-embryo experiments by adda-specific ELISA (Enzo-lifescience, Lausen, Switzerland). Samples were taken at the beginning of exposure from the freshly prepared exposure solutions, and at the end of the 96 h exposure from every beaker. ELISA was performed according to the user manual.

2.9 Statistics

Data were processed with GraphPad Prism 5 software and statistics were performed using One way ANOVA with Dunnett's post-test. For data processing of liver organ cultures, each liver was analysed separately and transcriptional changes after exposure to MC-LR and nodularin, respectively, were normalized to the respective control.

3. Results

3.1 Uptake of fluorescent labeled MC-LR into zebrafish eleuthero-embryos

Uptake and distribution was directly observed in zebrafish eleuthero-embryos by use of a fluorescently labelled MC-LR derivative. Exposure of chorionated early embryos to 1 $\mu\text{mol L}^{-1}$ of MC-LR labeled with Alexa 488 (Grundler et al., 2015) resulted in fluorescence accumulation on the surface of the chorion (Figure 2). Manual de-chorionation of the embryos clearly showed that the fluorescence was bound to the chorion and not the embryo itself, suggesting that the fluorescently labelled MC-LR derivative did not penetrate the chorion (data not shown).

In contrast, fluorescent MC-LR-Texas red, but also the fluorophore Texas red showed uptake into eleuthero-embryos after hatching. Exposure of zebrafish eleuthero-embryos to MC-LR labeled with Texas red (Grundler et al., 2015) resulted in a strong fluorescence signal within the glomerulus of the pronephros, but not in the liver (Figure 3). This is also demonstrated by cryo-sections taken from 7 days old zebrafish eleuthero-embryos exposed to fluorescent MC-LR from 72 to 168 hpf (Figures 3E, F). In addition, the fluorophore Texas red itself accumulated in the glomerulus (data not shown).

3.2 Transcriptional expression of *drOatp1d1*, *drOatp2b1* and *drOatp1f* in zebrafish early life stages

Our *in vitro* study (Faltermann et al., 2015a) indicated that uptake of MC-LR and nodularin is mediated by the uptake transporter *drOatp1d1*. We therefore evaluated whether this and other *drOatps* were expressed in different developmental stages of zebrafish from 24-168 hpf during embryonic development. Therefore, we analysed mRNA abundance of three Oatp isoforms. Transcripts were already measurable at 24 hpf and transcripts of *drOatp1d1* were detected in all development stages (Figure 4). Transcripts of *drOatp2b1* were even more abundant, while *drOatp1f* transcripts showed very low abundance (Figure 4A). For both *drOatp1d1* and *drOatp2b1*, the abundance increased after hatching at 72 hpf and remained at a higher level up to 168 hpf. Exposure of eleuthero-embryos to the solvent control (0.01 % DMSO) used for exposure to cyanobacterial toxins had no significant influence on the expression of these uptake transporters (Figure 4B). These data demonstrated the presence of transcripts of *drOatp1d1*, a gene encoding uptake transporter mediating uptake of MC-LR and nodularin. This also holds for *drOatp2b1*. Results are based on mRNA, and we cannot exclude that translation into functional transporter is different. Due to technical reasons we could not directly

compare the expression of *Oatps* with uptake of the toxins. However, uptake found by fluorescent labeled MC-LR may be based on the transporter *Oatp1d1*.

3.3 Transcriptional changes in liver organ cultures after exposure to MC-LR and nodularin

Freshly isolated livers from adult zebrafish were cultured *ex vivo* as organ cultures for a short period of time and exposed to MC-LR and nodularin, respectively, for 2.5 and 5 h. Exposure concentrations were 0.15 and 0.3 $\mu\text{mol L}^{-1}$ MC-LR, and to 0.5 and 1 $\mu\text{mol L}^{-1}$ nodularin, respectively. As positive control 0.5 $\mu\text{mol L}^{-1}$ thapsigargin was used. Induction of ER-stress, oxidative stress and additional genes was investigated by transcriptional changes. Key marker genes of ER-stress, including the chaperone *glucose regulated protein 78 (bip)*, the spliced form of *x-box binding protein (xbps-1s)*, the *CCAAT-enhancer-binding protein homologous protein (chop)* and *activating transcription factor 4 (atf4)* were determined. Nodularin led to a significant transcriptional induction of all genes involved in ER-stress, including *bip*, *xbps-1s*, *chop* and *atf4* after the 2.5 and 5 h exposures. Similarly, MC-LR led to induction of these genes at both exposure times with the exception of *atf4* (Figure 5A). Strongest up-regulation occurred with *chop*, and its protein is involved in ER-stress induced apoptosis. These data clearly suggest induction of ER-stress and associated UPR in the adult zebrafish liver.

Strong transcriptional changes also occurred for *tumor necrosis factor alpha (tnfa)* and *dual specificity phosphatase 5 (dusp5)* (Figure 5B). $\text{TNF}\alpha$ is one of the major pro-inflammatory signaling factors. After binding to its receptors, $\text{TNF}\alpha$ can induce numerous effects, including activation of MAPK signaling, that regulates proliferation, differentiation and apoptosis. Within the mitogen-activated protein kinase (MAPK) signaling *Dusp5* dephosphorylates kinases. Also this pathway was strongly induced at all nodularin and the high MC-LR concentration after 5 h exposure (Figure 5B).

In contrast, no alterations in transcript levels of pro-apoptotic genes *Bcl-2 like protein 4 (bax)* and *p53* occurred (Figure 5C). Moreover, no alteration in transcriptional expression of oxidative stress related genes *cat* and *gst* occurred (Figure 5C).

3.4 Transcriptional changes in zebrafish early life stages after exposure to MC-LR and nodularin

First, we determined the exposure concentrations of MC-LR and nodularin in the eleuthero-embryo exposures by ELISA. In the beginning ($t = 0$), concentrations were close to nominal. At nominal 0.1 and 1 μM MC-LR measured levels were 0.1 $\mu\text{mol L}^{-1}$ and 0.875 $\mu\text{mol L}^{-1}$, respectively. In case of 0.1 and 1 $\mu\text{mol L}^{-1}$ nodularin, measured concentrations were 0.08 $\mu\text{mol L}^{-1}$ and 1.325 $\mu\text{mol L}^{-1}$, respectively. During the 96 h exposure, MC-LR concentrations were 0.05 and 0.43 $\mu\text{mol L}^{-1}$, respectively, which is about 50 % of the initial concentration. No decrease was observed for nodularin during the 96 h exposure. The concentrations were 0.12 and 1.303 $\mu\text{mol L}^{-1}$, respectively. The solvent controls contained no MC-LR or nodularin. For easier comparison of effects data, nominal concentrations are used in this work.

We analyzed whether MC-LR and nodularin induce similar transcriptional alterations in zebrafish eleuthero-embryos as in liver organ cultures. We determined the expression of transcripts of genes involved in ER-stress response, such as *bip*, *xbps-1*, *chop*, and *atf4* at different development stages. Figure 6A shows that the 4 h exposures at both 120 and 168 hpf did not result in significant transcriptional alterations. Long-term exposures of 24 h and 48 h (Figure 6B), as well as 96 h (Figure 6C), which started after embryos have hatched (at 72 hpf), did not lead to significant transcriptional alterations. Thus, no changes in expression of any of the selected target genes involved in ER-stress occurred after exposure to 0.1 and 1 $\mu\text{mol L}^{-1}$ MC-LR and nodularin, respectively. This contrasted to data in zebrafish adult liver. The positive control thapsigargin led to a significant up-regulation of *bip*, *xbp-1s* and *chop*, which demonstrates the reactivity of eleuthero-embryos.

Furthermore, transcripts of genes involved in oxidative stress response (*cat*, *gst*) and apoptosis (*bax*, *p53*) showed no significant alterations compared to control after long-term exposures (Figures 7A, 7B).

4. Discussion

In our present study, we demonstrated the uptake and accumulation of MC-LR in the glomerulus of the pronephros of zebrafish eleuthero-embryos. Transcripts of *Oatps*, including *drOatp1d1* that, if

translated to protein and integrated in the cell membrane, mediate the active uptake of MC-LR and nodularin, were expressed at 24 hpf and increased after hatching in zebrafish embryos. We also demonstrated that MC-LR led to changes in transcripts of genes indicative of ER-stress, similar to the ones in our previous study in a human hepatoma cell line (Christen et al., 2013). Moreover, we showed for the first time that nodularin, like MC-LR, induced changes in mRNA abundance that suggest ER stress induction. Although a quantitative comparison of the transcriptional changes induced by MC-LR and nodularin is not possible due to the different concentrations of the two toxins we used in our study, our results nevertheless suggests a novel and similar transcriptional mode of action of these heptapeptides in fish. In contrast to the adult liver, no indication of ER stress was detectable in eleuthero-embryos.

4.1 Expression of *Oatps* and uptake

Several organic anion transporting polypeptides (Oatp) or solute carriers are known to function in xenobiotic transport in mammals. However, little is known about their occurrence and function in fish. Popovic et al., (2010) reported on the transcriptional occurrence of several *Oatps* in adult zebrafish and characterized the substrate specificity of Oatp1d1 (Popovic et al., 2013). Oatps are apical or basolaterally located in tissues and can serve as transporters for endogenous compounds as well as xenobiotics. They can also be inhibited through contaminants, thus affecting the distribution of endogenous substances like bile acids and steroids. In our present work, we described for the first time the occurrence of transcripts of three *Oatps* in zebrafish early life stages. Two *Oatp* transcripts, *drOatp1d* and *Oatp2b1*, were found expressed at 24 hpf. The abundance increased after hatching and remained at a more or less equal level up to 168 hpf. Both genes encoding the transporters are highly expressed in the adult liver, whereas *drOatp1f* is mainly expressed in the kidneys (Popovic et al., 2010). Besides liver, high mRNA levels of *Oatp2b1* have been found in gills of zebrafish (Popovic et al., 2010). In our associated work, we demonstrated that drOatp1d1 mediates the uptake of MC-LR and nodularin (Faltermann et al., 2015a). . In rainbow trout, Oatp1d1 was also shown to be an uptake transporter for MC-LR (Steiner et al., 2014).

Microcystin and nodularin are both hepatotoxins, and due to high expression of Oatp uptake transporters in the liver, these toxins accumulate mainly in this organ in adult fish. By use of fluorescent MC-LR we expected accumulation of fluorescence signal also in the liver of zebrafish eleuthero-embryos. However, we provided direct evidence that MC-LR accumulated in eleuthero-embryos within the glomerulus of the pronephros (Figure 4). We did not find fluorescence in the liver in this early life stage.

4.2 ER-stress, TNF- α induction and influence on MAPK pathway in the liver but not in eleuthero-embryos

In mice, ER-stress induction was reported in the liver and kidneys after intraperitoneal injection of MC-LR (Qin et al., 2010). Similarly, induction of ER-stress by microcystin-LR was demonstrated on mRNA and protein level in human hepatoma cells (Christen et al., 2013). In our present study, increasing mRNA levels of four selected ER-stress markers were found in adult zebrafish liver organ cultures after exposure to microcystin-LR. We also demonstrated for the first time that nodularin exposure resulted in changes in mRNA abundance of the selected transcripts that suggest ER-stress induction. Already after 2.5 h of exposure, and even more prominent after 5 h of exposure, transcripts of all key marker genes, including *bip*, *xbp-1s*, *chop* and *atf4* were significantly induced. However, results are on mRNA level and need not necessarily have consequences on protein level. Induction of *bip* on protein level after exposure to MC-LR was shown not only by Christen et al., (2013) in human hepatoma cells, but also in the liver of adult medaka after oral gavage of an extremely high concentration of 5 mmol L⁻¹ MC-LR (Malécot et al., 2009).

Another natural toxin, okadaic acid, which is produced by dinoflagellates and also acts as an inhibitor of serine/threonine phosphatases is taken up by diffusion and influenced protein processing in the ER in liver of adult zebrafish. Furthermore, MAPK signaling was influenced, as transcriptional changes of *dual specificity phosphatase 5 (dusp5)* has also been found in the same study (Zhang et al., 2014). Similarly, we found strong transcription induction of *dusp5* in zebrafish liver organ culture after exposure to MC-LR and nodularin (Figure 5B). In addition, *tumor necrosis factor alpha (tnfa)* was induced (Figure 5B). TNF α interacts with mitogen-activated protein kinases (MAPK) signaling

(Wajant et al., 2003). Provided that the transcriptional changes are reflected in corresponding changes in the function of encoded proteins, this might have an influence not only on cell proliferation, but also on apoptosis (Wada and Penninger, 2004). TNF α is known as an endogenous tumor promoter. It belongs to the group of pro-inflammatory cytokines involved in the pathogenesis of several diseases including cancer (Wajant et al., 2003). Therefore, the induction of TNF α and MAPK signaling may contribute to the tumor promoting effect of MC-LR and nodularin.

In contrast to the adult liver, no significant transcriptional changes in ER-stress genes were detected in MC-LR and nodularin exposed eleuthero-embryos (Figure 6). This confirms previous studies in zebrafish eleuthero-embryos exposed to 0.1 and 1 $\mu\text{mol L}^{-1}$ MC-LR for 96 h (Rogers et al., 2011). In this microarray study, no significant changes in the transcription of ER-stress marker genes *bip*, *xbp-1s*, *chop* and *atf4* were found.

The difference in the reaction of the adult liver and eleuthero-embryos found in our present study is interesting. Although we cannot discount the possibility that the regulation in embryos and adults is different, it is more likely that our results are based on tissue differences and their dilutions: Our uptake experiment indicated uptake and accumulation of MC-LR in the pronephros of eleuthero-embryos. If ER-stress induction is restricted to specific tissues such as liver and pronephros, our technique applied appears not sensitive enough. Our RT-qPCR analysis was based on RNA extracted from the whole body of eleuthero-embryos and not the liver or pronephros alone; thus it seems not sensitive enough to detect changes in a specific tissue or organ. Thus, the signal of a specific organ became diluted in the whole body analyses, and the total of alterations of ER-stress related gene transcripts become too low for detection. However, the suitability of this approach to detect strong ER-stress in eleuthero-embryos was demonstrated by our data resulting from the known ER-inducer thapsigargin (Hu et al., 2006). In our experiments thapsigargin, a strong ER-stress inducer, resulted in strong inductions of *bip*, *xbp-1s* and *chop* in eleuthero-embryos (Figure 6 B). To detect ER-stress in specific tissues in zebrafish eleuthero-embryos, in-situ-hybridization could be a sensitive method to be considered in further experiments. It is also important to note that ER-stress or apoptosis should be confirmed on the protein level in forthcoming experiments.

Furthermore, uptake of MC-LR is low in early life stages (Wang et al., 2005), thus only little effects of waterborne exposures are expected to occur. Since nodularin and microcystin are structurally similar, a low uptake is also assumed for nodularin. The chorion protects the early embryo as shown by fluorescent MC-LR (MC-LR-alexa488) (Figure 2). Previously reported expressional changes in hatched eleuthero-embryos (Li et al., 2009; Yan et al., 2012) could not be verified in our study (data not shown). Only when MC-LR was injected into embryos, marked effects occurred (Huynh-Delerme et al., 2005). The main uptake route of MC-LR is thought to occur via the gastrointestinal tract. Since zebrafish start feeding at 144 - 168 hpf (Strähle et al., 2012), significant uptake of water-borne cyanotoxins is only expected at this development time.

Oxidative stress induction and apoptosis by MC-LR was reported in aquatic animals (Amado and Monserrat, 2010; Nong et al., 2007). However, in our experiments, there was also no indication of oxidative stress induction or apoptosis in the adult liver and eleuthero-embryos. This was indicated by the lack of transcriptional changes of *cat* and *gst*, as well as pro-apoptotic genes *bax* and *p53*. Our concentrations were too low for inducing this reaction. Only at higher levels of 5 $\mu\text{mol L}^{-1}$ MC-LR, induction of *bax* and *p53* transcription was previously reported in eleuthero-embryos (Zeng et al., 2014).

Typically, environmental concentrations of MC-LR are below 0.1 $\mu\text{mol L}^{-1}$ (100 $\mu\text{g L}^{-1}$), but can reach up to 1 $\mu\text{mol L}^{-1}$ (1000 $\mu\text{g L}^{-1}$) (Sivonen and Jones, 1999; Welker et al., 2005) or even more (WHO, 2011). Therefore, our exposure concentrations can be considered as environmentally realistic. This leads to the conclusion that effects of MC-LR and nodularin to embryos and eleuthero-embryos of fish at environmental levels are rather low. However, in adult fish, the liver is affected via important transcriptional modes of action.

In summary, our study demonstrates that MC-LR and nodularin are similar in their effects on the liver of adult fish. They lead to transcriptional changes that suggest induction of ER-stress and associated UPR, TNF α and MAPK pathways. These mechanisms may contribute to the tumor-promoting effect of MCs and nodularin in animals. Both toxins induced transcriptional changes indicating ER-stress, which also occurs in human hepatoma cells (Christen et al., 2013). However, despite transcriptional expression of *Oatps* and accumulation in the glomerulus of the pronephros, no

effects were detectable in eleuthero-embryos. Further studies should reveal the reasons for the lack of detectable expressional changes in this life stage.

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Figure Legends.

Figure 1. Overview of the different experimental designs for the MC-LR (A) and Nodularin (B) exposure of ZF early life stages, as well as for the uptake experiments of fluorescent labeled MC (C) and the sampling of embryos for *Oatp* expression analysis (D).

Figure 2. Microscopic image of a zebrafish control embryo (A), the respective fluorescent image (B), and the fluorescent image of a 24 hpf embryo exposed to $1 \mu\text{mol L}^{-1}$ fluorescent MC-LR-Alexa 488 for 24 h (C).

Figure 3. Image of zebrafish eleuthero-embryos exposed to $1 \mu\text{mol L}^{-1}$ fluorescent MC-LR-Texas red and corresponding fluorescent images (A, B: magnification 10x, C: magnification 20x) and cryosections of control (D) and fluorescent MC-Texas red-exposed eleuthero-embryo (E, F) at 168 hpf (D, E: magnification 10x, F: magnification 20x). Confocal image of the glomerulus of fluorescent MC-Texas red exposed 168 hpf eleuthero-embryo (G).

Figure 4. Expression of *drOatp1d1*, *drOatp2b1* and *drOatp1f* mRNA in early developmental stages of zebrafish (A) and comparison of *drOatp1d1* and *drOatp2b1* expression in zebrafish embryos raised in fish water or in fish water containing 0.01 % DMSO from 72 hpf on (B). Values are presented as mean $\pm$ SEM.

Figure 5. Alteration of transcripts (mRNA) of genes involved in ER-stress (A), mitogen activated protein kinase (MAPK) signaling (B), oxidative stress and apoptosis (C) in liver organ cultures of female zebrafish after exposure to 0.15 and $3 \mu\text{mol L}^{-1}$ MC-LR, and 0.5 and $1 \mu\text{mol L}^{-1}$ nodularin, respectively. Shown is also the positive control for ER-stress induction ($1 \mu\text{M}$ thapsigargin). Results are from two independent experiments with both MC-LR and nodularin exposures. Values are

presented as mean $\pm$ SEM. Significant changes in transcript levels compared to solvent control (0.001 % DMSO) are indicated by asterisks. (* $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$).

Figure 6. Transcript levels of genes involved in ER-stress after short-term exposure of 4 h at 120 hpf and 168 hpf (A), and after long-term exposure of 24 h and 48 h (B) or 96 h (C) of 72 hpf zebrafish eleuthero-embryos to 0.1 $\mu\text{mol L}^{-1}$ and 1 $\mu\text{mol L}^{-1}$ MC-LR, nodularin and to the positive control for ER-stress induction (1 $\mu\text{mol L}^{-1}$ thapsigargin). Values are presented as mean $\pm$ SEM.

Significant changes in gene expression compared to solvent control are indicated by asterisks. (* $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$).

Figure 7. Expression of transcripts of genes involved in oxidative stress response and apoptosis after long-term exposure of 24 h, 48 h (A) or 96 h (B) of 72 hpf zebrafish eleuthero-embryos after exposure to 0.1 $\mu\text{mol L}^{-1}$ and 1 $\mu\text{mol L}^{-1}$ MC-LR and nodularin. Values are presented as mean $\pm$ SEM. Significant changes in gene expression compared to solvent control are indicated by asterisks. (* $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$).

Tables

Table 1. Primer sequences used for Quantitative Real-Time PCR Analysis

Target gene	Primer	Sequence (5' - 3')
<i>rpl13a</i>	Forward	AGC TCA AGA TGG CAA CAC AG
	Reverse	AAG TTC TTC TCG TCC TCC
<i>bip</i>	Forward	CGA AGA AGC CAG ATA TCG ATG A
	Reverse	ACG GCT CTT TTC CGT TGA AC
<i>xbp-1s</i>	Forward	TGT TGC GAG ACA AGA CGA
	Reverse	CCT GCA CCT GCT GCG GAC T
<i>chop</i>	Forward	GAG TTG GAG GCG TGG TAT GA
	Reverse	CCT TGG TGG CGA TTG GTG AA
<i>atf4</i>	Forward	CTT TCT CTC CTC CTG CTT CT
	Reverse	GAG TCA CAC GAC CCA ATC A
<i>cat</i>	Forward	AGG GCA ACT GGG ATC TTA CA
	Reverse	TTT ATG GGA CCA GAC CTT GG
<i>gst</i>	Forward	CTA TAC ATG CGG CGA AGC T
	Reverse	GGC ATT GCT CTG GAC GAT
<i>bax</i>	Forward	ACA GGG ATG CTG AAG TGA CC
	Reverse	GAA AAG CGC CAC AAC TCT TC
<i>p53</i>	Forward	GCT TGT CAC AGG GGT CAT TT
	Reverse	ACA AAG GTC CCA GTG GAG TG
<i>tnfα</i>	Forward	ACC AGG CCT TTT CTT CAG GT
	Reverse	TGC CCA GTC TGT CTC CTT CT
<i>dusp5</i>	Forward	TGA AGG TCT CCA GCA TAG
	Reverse	GGA ATG ACG AAC TGT AGA G

ER stress, oxidative stress

Uptake

Figure 2

Figure 3

Figure 4

Figure 5.

Figure 6

Figure 7.