

## MicroRNA expression in liver of whitefish (*Coregonus lavaretus*) exposed to microcystin-LR

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### ABSTRACT

MicroRNAs (miRNAs) are small, highly conserved, non-coding RNAs that regulate gene expression of target mRNAs through cleavage or translational inhibition. In the field of toxicology, the relationship between toxicity and microRNA expression is poorly understood. In the present study we analyzed the abundance of 9 selected miRNAs (omy-miR-21, omy-miR-21t, omy-miR-122, omy-miR-125a, omy-miR-125b, omy-miR-125t, omy-miR-199-5a, omy-miR-295, omy-let-7a) and mRNA of 3 genes (histone H2A, ribosome protein rpl19, and Dicer which is a miRNA processing enzyme) in liver samples of whitefish exposed to Microcystin-LR (MC-LR) at a dose of 100 µg·kg<sup>-1</sup> body weight for 24 or 48h. In the examined liver tissue, omy-miR-122 showed the highest relative constitutive level, what is

consistent with data obtained from fish and mammals. Unexpectedly, the reference H2A mRNA level was consistently up-regulated (over 20-fold;  $P < 0.05$ ) in fish liver after both 24 and 48h of exposure to MC-LR. The result may suggest that MC-LR acts as an initiator of specific cell-physiologic signals triggering DNA replication in fish liver cells. MC-LR treatment had no effect on the examined miRNAs levels, except for omy-miR-125a and omy-let-7a. Whereas omy-miR-125a was up-regulated (ER=2.68; S.E. 1.61-6.78;  $P < 0.05$ ), omy-let-7a was down-regulated (ER=0.55; S.E. 0.32-0.79;  $P < 0.05$ ) in whitefish liver after 48h of the treatment with MC-LR, when compared to controls. More work with the fish is essential for understanding the crosstalk of the regulatory network controlled by the two miRNAs in the context of MC-LR toxicity.

### INTRODUCTION

Current literature on expression patterns of tissue or developmental stage specific genes and transcription factors suggest global but tight posttranscriptional regulation. MicroRNAs (miRNAs) are small non-coding regulatory RNAs (19-23 nucleotides) that bind to recognition sequences on 3'-untranslated regions (3'-UTRs) of mRNAs and target them for degradation, translational repression, or miRNA mediated mRNA decay (Zhang et al. 2007). In metazoans miRNA complementarity to their targets is far from perfect, so one miRNA can bind up to 200 targets, and each mRNA could have recognition sites for more than one miRNA (Wienholds and Plasterk 2005). It is estimated that about 30% of the human protein-coding genes are negatively regulated by miRNA (Lewis et al. 2005), which suggests that miRNAs are very important regulators of gene expression process (Zhang et al. 2007).

Although specific functions and target mRNAs have been assigned to only a few dozen miRNAs, much experimental evidence suggests that miRNAs participate in the regulation of

a vast spectrum of biological processes. miRNAs control diverse cellular processes including animal development and growth (Le et al. 2009; Ramachandra et al. 2008), cell differentiation (Chen et al. 2006), signal transduction (Mudhasani et al. 2008), cancer (Jerome et al. 2007), neuronal disease (Bicker and Schrott 2008; Gregory et al. 2004), virus-induced immune defense (Lecellier et al. 2005), programmed cell death (Cimmino et al. 2005), insulin secretion (Poy et al. 2004), and metabolism (Esau et al. 2006). Understanding of RNA interference (RNAi) has been made possible through a variety of experimental approaches using different model organisms (Bernstein et al. 2001), including fish (Flynt et al. 2009).

Microcystins (MCs) are a family of extensively studied cyclic heptapeptide hepatotoxins among which MC-LR is both the most toxic and the most commonly encountered variant (Mirura et al. 1989), which is produced by some cyanobacteria genera such as *Microcystis*. The adverse effects of MC-LR are closely related to oxidative stress processes, free radicals formation and DNA damage occurrence, and all involve major gene transcript changes (e.g. Chen et al. 2005; Weng et al. 2007). Whereas a

number of studies, predominantly on mammals, have been done so far to analyze the effect of MC-LR on pathological alterations in cells and tissues at the gene or protein level, very little is known about the changes in fish, including the regulation of the transcriptional and post-transcriptional program of fish tissues in response to MC-LR exposure. Alterations to cellular miRNA expression profiles would represent a novel mode of action of MC-LR exposure, and specific alterations in miRNA expression may be a powerful biomarker for these toxins with serious effects on fish and human health.

In mammals, several microRNAs associated with stress response have been reported (Pillai et al. 2007). To ensure robust and precise response to cellular signals, expression of the stress related genes is tightly regulated by these miRNAs from the transcriptional to the post-translational levels. However, in the field of toxicology, the relationship between toxicity and microRNA expression is poorly understood. There are only few reports concluding that miRNAs may be key molecules involved in aberrant gene expression in liver cells. For example, Fukushima et al. (2007) have shown that two well known hepatotoxicants which induce hepatocellular injuries and necrosis, acetaminophen (APAP) or carbon tetrachloride (CCL<sub>4</sub>), were capable of modulating expression of two miRNAs (miR-298 and miR-370) in rats, and that these effects were accompanied by impaired liver metabolism. The observation that miRNAs levels were changed by hepatotoxicants in rats prompted us to investigate the role of fish microRNAs in the context of MC-LR toxicity. In the present study, we analyzed the abundance of 9 selected miRNAs in liver samples of whitefish exposed to MC-LR for 24 or 48h. In addition, we obtained partial sequence and examined expression of Dicer, a specialized ribonuclease that is required for producing mature, single-stranded miRNA molecules (Wienholds et al. 2003).

## MATERIAL AND METHODS

### Preparation of samples

In 2008, we began a study of microcystin-LR induced transcriptional changes in European whitefish, *Coregonus lavaretus* L. (Brzuzan et al. 2009). A treatment was carried out on hatchery reared fish (75.6±8.0g mean weight, 21.2±0.8cm mean length). The fish were treated in accordance with the rules conforming to principles of Laboratory Animal Care, NIH publication No. 86-23, revised in 1985). Fish were injected with different concentrations of MC-LR (0, 10 or 100µg·kg<sup>-1</sup> of body weight) and then sacrificed at either 0, 8, 24, 48 or 72h later, and their liver tissue were harvested and stored (-80°C) in a nucleic acid preserving solution, RNALater™ (Qiagen, Hilden, Germany). See Brzuzan et al. (2009) for more methodological details of the experiment.

### Real-Time qPCR of miRNAs, H2A and Dicer

Total RNA from previously preserved liver tissues of control and treated (100µg·kg<sup>-1</sup> of body weight) whitefish (n=3), collected at 24 and 48h of the experiment, was isolated using Total RNA Mini isolation kit (A&A Biotechnology, Gdynia, Poland) followed by DNase treatment. A one-year long tissue storage had no effect on total RNA and mRNA integrity, as verified by RNA electrophoresis and accurate A<sub>260</sub>/A<sub>280</sub> ratios (data not shown).

The expression of miRNAs, H2A and Dicer was measured using miScript PCR System (Qiagen). 1µg of DNase-treated RNA was converted to cDNA using miScript reverse transcriptase mix (miScript Reverse

**Table 1. Primers used for Real-Time qPCR analysis.**

MicroRNA	Sequence (5'-3')	Real Time Primer Sequence (5'-3')	Primer length (bp)	Source
omy-miR-21	UAGCUUAUCAGACUGGUGUUGGC	TAGCTTATCAGACTGGTGTGTTGGC	23	Ramachandra et al. (2008)
omy-miR-21t	UAGCUUAUCAGACUGGUGUUGAC	TAGCTTATCAGACTGGTGTGTTGAC	23	Ramachandra et al. (2008)
omy-miR-122	UGGAGUGUGACAAUGGUGUUU	TGGAGTGTGACAATGGTGTTTT	21	Salem et al. (2009)
omy-miR-125a	UCCCUGAGACCCUUAACCUGUG	TCCCTGAGACCCCTAACCTGTG	22	Ramachandra et al. (2008)
omy-miR-125b	UCCCUGAGACCCUUAACUUGUGA	TCCCTGAGACCCCTAACTTGTGA	22	Ramachandra et al. (2008)
omy-miR-125t	UCCCUGAGACUUUAACUUGUGA	TCCCTGAGACTTTAACTTGTGA	22	Ramachandra et al. (2008)
omy-miR-199-5a	CCCAGUGUUCAGACUACCUGUUC	CCCAGTGTTCAGACTACCTGTTC	23	Salem et al. (2009)
omy-miR-295	ACAGUACACUACUUUUGA	ACAGTACACTACTTTTGA	18	Salem et al. (2009)
omy-let-7a	UGAGGUAGUAGUUUGUGUCGUC	TGAGGTAGTAGTTTGTGTGTCGTC	22	Salem et al. (2009)
Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon length (bp)	Source
histone H2A	TCCCAAGAAGACTGAGAAGG	TTTGTGAGCTAGGTGGTTGG	113	Ramachandra et al. (2008)
rpl19	AGGCACACAGGAATTGGTAAGAG	CGCATCCAGGACAGCTTCTC	71	Brzuzan et al. (2009)
Dicer	AGGAGGCAGTGCTACCCATAA	AAGTTGAGTTCGTCAGGCAGA	128	Ramachandra et al. (2008)

Transcription kit; Qiagen), a blend of enzymes comprising poly(A) polymerase and reverse transcriptase, and a mixture of oligo-dT (containing universal tag sequence), according to the manufacturer's recommendations. The cDNA was then used for Real-Time PCR quantification of miRNAs (using a miRNA target-specific primer and the miScript Universal Primer (Qiagen), or H2A and Dicer RNA (using gene-specific primers). miRNA target-specific and gene specific primers for Real-Time qPCR assay are shown in Table 1.

Real-Time qPCR was performed on a ABI 7500 Real-Time PCR System (Applied Biosystems; Branchburg; USA). For quantitation of miRNAs, each 50 $\mu$ l of Real-Time PCR reaction mixture consisted of 2x QuantiTect SYBR Green PCR Master Mix (Qiagen), 5 $\mu$ l of miScript Universal Primer (Qiagen), 5pmol of miRNA specific primer and 2 $\mu$ l of cDNA as a template. For the examination of H2A and Dicer, gene specific primers were used instead of the Universal Primer (Table 1). mRNA level of endogenous rpl19 was used to normalize the amounts of Dicer mRNA. All Real-Time PCR reactions were performed as follows: 95°C for 15min, then 45 cycles of 94°C for 15s, 55°C for 30s and 72°C for 40s. cDNA samples of each experimental group of fish (Table 1) were analyzed in duplicate in the singleplex mode. On the plate, negative water controls (NTCs) and genomic DNA control were included to rule out the possibility of PCR amplification resulting from upstream contamination. For quality check of both H2A and Dicer products, a melting curve analysis was performed after each run, and the specificity of all PCR products was documented by standard 2% agarose gel electrophoresis. Finally, representative H2A and Dicer amplicons were sequenced (Genomed) and the results were compared to known sequences in GenBank (BLAST; NCBI-NIH). The partial sequences of the whitefish H2A and Dicer mRNA were deposited in GenBank under accession Nos. HQ173701 and HQ173702, respectively.

Expression ratio (ER) of each miRNA or Dicer relative to endogenous control (H2A or rpl19) was computed as explained in our recent paper (Woźny et al. 2008). The calculations were based on single gene individual Real-Time PCR efficiency (E), and the threshold cycle difference ( $\Delta$ Ct) of a sample versus a control ( $\Delta$ Ct<sub>control-sample</sub>) according to the mathematical model: Ratio (ER) =  $[(E_{\text{target}})^{\Delta\text{Ct}_{\text{target}}}] / [(E_{\text{reference}})^{\Delta\text{Ct}_{\text{reference}}}]^{-1}$  given by Pfaffl (2001). Statistical differences among miRNA constitutive levels in liver were tested using analysis of variance (ANOVA) followed by Tukey's *post hoc* multiple comparison test. Before analysis the ER values were log-transformed and tested for normal distribution (Shapiro-Wilk W test) and for homogeneity of variance (Levene's test). Differences in miRNA, H2A or Dicer expression between control and treated samples were assessed in group means for statistical significance by randomization tests with REST© 2008 software (Pfaffl et al.

2002). In this study, 5000 randomizations were performed against time of the experiment.

## RESULTS AND DISCUSSION

### Analysis of whitefish histone H2A mRNA similarity and expression

The replication-dependent histone genes are the class of genes that provide the massive amounts of histones required for chromosome packaging during the S-phase of cell cycle. Those encoding the four core histones (H2A, H2B, H3 and H4) and the linker histone H1 are unique in that they do not contain introns, and that their mRNAs end in a highly conserved stem-loop structure instead of a poly(A) tail (Dominski and Marzluff 2007; Gilmartin 2005; Jaeger et al. 2005). This is why histone H2A (and others of this class) mRNA levels have been used successfully as endogenous controls in miRNA expression studies (e.g. Ramachandra et al. 2008). As histone synthesis outside the S-phase is highly toxic to the cell, multiple levels of regulation are involved to restrict histone production exclusively to the S-phase (Xu and Huang 2009). Unexpectedly in the present study, non-normalized H2A mRNA was consistently up-regulated in the liver of fish exposed to MC-LR. Therefore, to confirm the H2A amplicons to have been indeed products of the gene, they were sequenced and analyzed. Sequencing of the PCR amplicons revealed its high similarity to rainbow trout sequence containing both H2A coding region and H2A 3' non coding sequence (Figure 1A).

Due to the instability of H2A transcripts, in further analysis of miRNA expression the expression of omy-miR-122 was used as a reference. Once the expression data of H2A were normalized to the omy-miR-122, the up-regulation of H2A expression between control and treated sample, at 24 or 48h, was 21-fold (S.E. 1.17-174.46;  $P < 0.05$ ) or 27-fold (S.E. 6.76-103.40;  $P < 0.05$ ), respectively (Figure 1B). The apparent increase of the H2A mRNA in MC-LR treated whitefish, which is a hallmark for the DNA synthesis phase of cell cycle, would suggest that MC-LR acted as an initiator of specific cell-physiologic signals triggering DNA replication in liver cells. This result, however, is in contrast with the evidences that microcystins are capable rather of inducing several defense programs in exposed animal cells that are opposite to DNA synthesis, such as apoptosis (Chen et al. 2005) or cell cycle arrest (Žegura et al. 2003). As very little is known about the regulation of cell cycle clock program in fish cells, the way in which the cell division is controlled in fish tissues in response to MC-LR needs to be further tested experimentally (e.g. by BrdU incorporation, DNA content-mg<sup>-1</sup> liver). When confirmed, the expression of H2A gene would be indicative of liver-specific response to MC-LR toxicity.

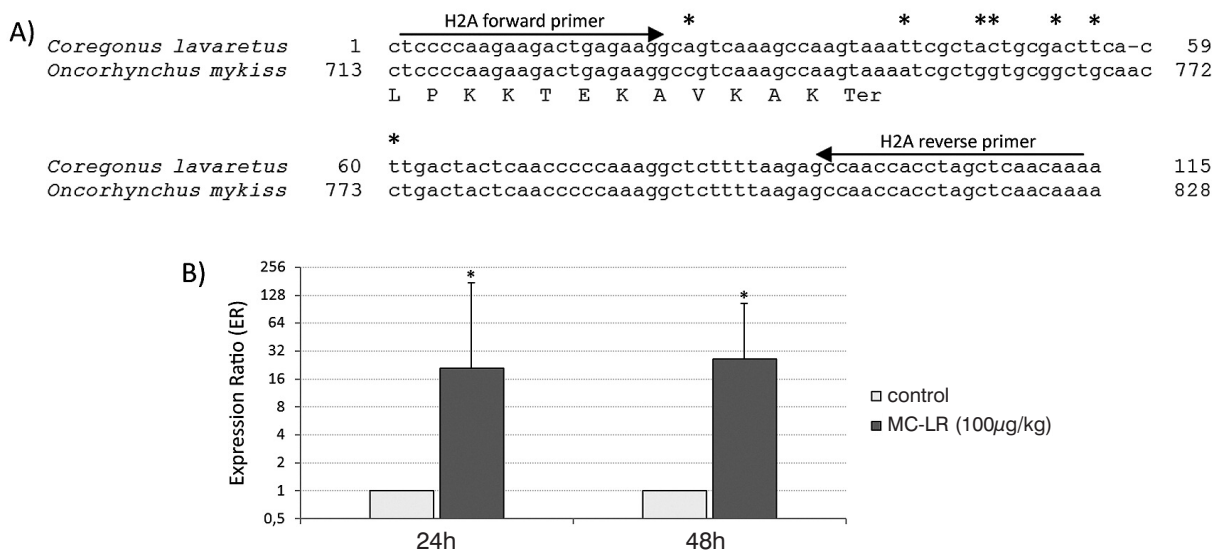


Figure 1. Whitefish histone H2A mRNA similarity and expression. (A) The nucleotide sequence and deduced amino acid sequence of whitefish histone H2A PCR amplicon (GenBank acc. No. HQ173701) aligned with rainbow trout H2A gene (Connor et al. 1984). Asterisks indicate variable nucleotide positions, while hyphen denotes an indel. The Real-Time PCR primers are indicated. (B) Histone H2A mRNA expression in control and treated whitefish ( $100\mu\text{g MC-LR}\cdot\text{kg}^{-1}$  body weight) exposed for 24 and 48h ( $n=3$ ). The bars represent mean values of expression ratios ( $\text{ER}\pm\text{S.E.}$ ), normalized by omy-miR-122. Data were analyzed using randomization procedure (REST© 2008). Asterisks indicate groups that showed significantly higher levels of H2A mRNA over a respective control group ( $P<0.05$ ).

### Analysis of whitefish Dicer similarity and expression

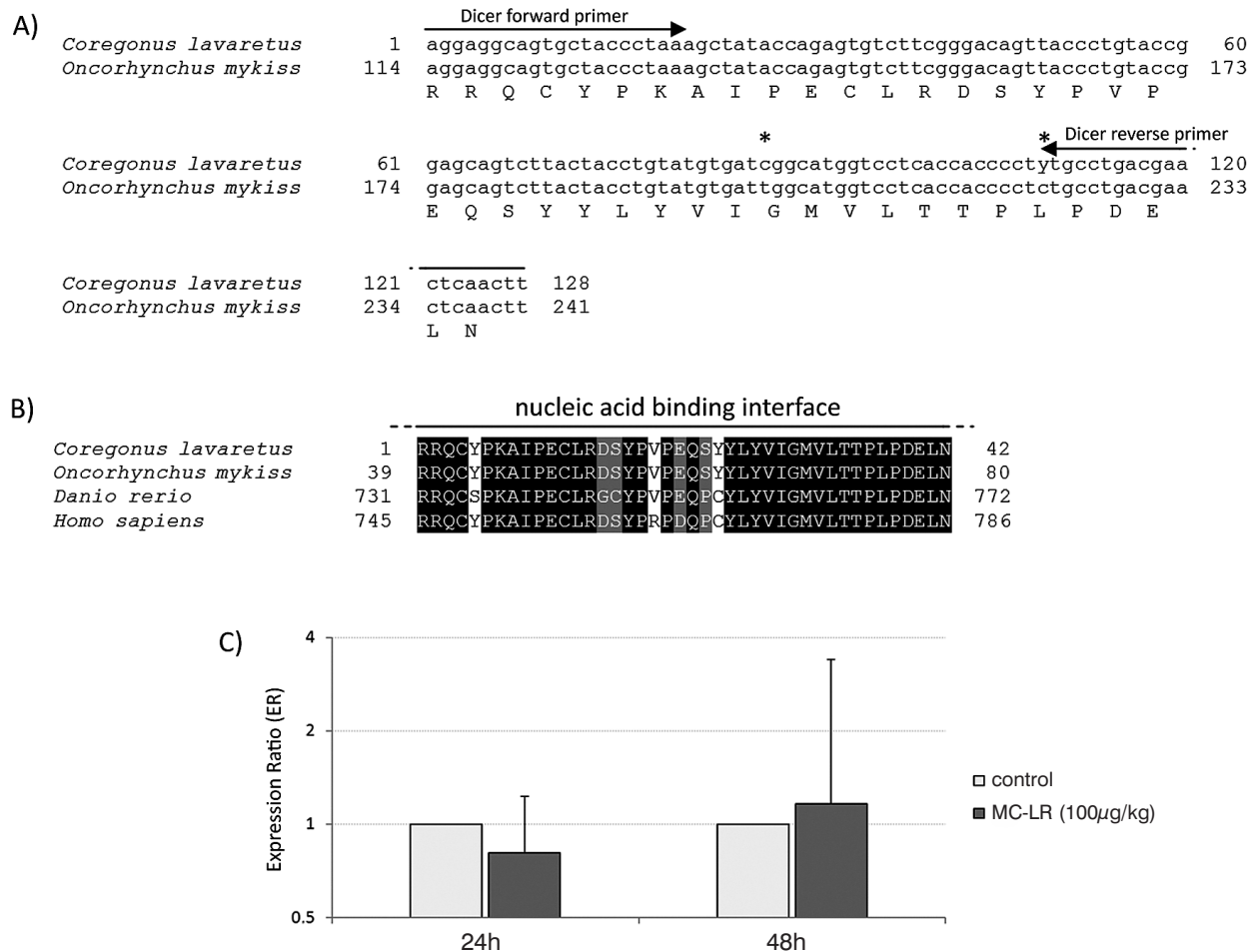
Dicer is a RNase III enzyme with two catalytic subunits involved in processing of all miRNAs (Bernstein et al. 2003; Yang et al. 2005). To confirm the obtained whitefish Dicer, PCR amplicons to have been indeed a Dicer gene expression product, two criteria were tested. First, sequencing of the PCR amplicon revealed its high similarity to rainbow trout cDNA containing Dicer coding sequence (Figure 2A). Secondly, deduced protein sequence alignment of the putative Dicer sequences of whitefish, rainbow trout, zebrafish, and human showed good homology with nucleic acid-binding interface (Figure 2B). The interface is localized close to PAZ domain which has been found crucial for nucleic acid-binding, with a strong preference for single-stranded nucleic acids (RNA or DNA) or RNA duplexes with single-stranded 3' overhangs (Song et al. 2003). The specificity of Real-Time PCR amplification for the Dicer gene was further confirmed by the melting curve analysis and resulted in single product specific melting temperature,  $79.5^{\circ}\text{C}$ . Gel electrophoresis revealed the presence of single product at the expected lengths (Table 1).

Dicer mRNA levels in MC-LR treated whitefish were approximately equal with those of control fish, after both 24 and 48h of the experiment (Figure 2C). This may indicate that Dicer was not influenced by the treatment, during the studied period.

### Profiles of miRNAs in whitefish liver

Quantifying miRNAs in different tissues is an important initial step to investigate functions of miRNAs. Tissue distribution of miRNAs provides essential baseline references to analyze variation of miRNA expression under various physiological conditions. To determine the expression levels of 9 selected miRNAs in the liver (Figure 3) we performed Real-Time qPCR. The data were normalized to omy-miR-295, the least abundant miRNA examined in the study ( $\log\text{ER}=0.00$ ). omy-miR-122 showed the highest relative abundance ( $\log\text{ER}=8.77$ ; S.E. 8.27-9.37;  $P<0.05$ ). The prominent expression of omy-miR-122 (Figure 3) in the liver of whitefish is consistent with data from fish (Salem et al. 2009) and mammals (Ason et al. 2006). It is the most abundant miRNA in human liver, silencing of which results in a modulation of expression of several hundred genes (Girard et al. 2008; Krutzfeldt et al. 2005).

Two other miRNAs, omy-miR-21 and omy-miR-125b, were only slightly less abundant in liver than miR-122 (Figure 3). The relatively high abundance of miR-21 in whitefish liver ( $\log\text{ER}=6.93$ ; S.E. 6.47-7.52;  $P<0.05$ ) is not a surprise as this miRNA accounts for almost 35% of all clones isolated from rainbow trout miRNA library (Salem et al. 2009) and for 40% in that of zebrafish (Chen et al. 2005). It is suggested that miR-21 plays important and housekeeping cellular roles; differentiation and carcinogenesis (Fujita et al. 2008), and



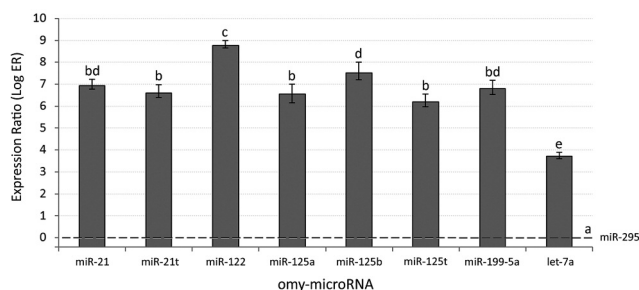
**Figure 2.** Whitefish Dicer mRNA similarity and expression. (A) The nucleotide sequence and deduced amino acid sequence of whitefish Dicer PCR amplicon (GenBank acc. No. HQ173702) aligned with rainbow trout Dicer coding sequence (AY523839). Asterisks denote variable nucleotide positions. The Real-Time PCR primers are indicated. (B) Multiple alignment of the deduced amino acid sequence of whitefish Dicer using homologous sequences of rainbow trout (AY523839), zebrafish (NP\_001154925.1) and human (NP\_085124.2). The alignment reveals that the putative Dicer of whitefish has a domain, nucleic acid-binding interface, which shows good homology with those of trout, zebrafish and human. (C) Dicer mRNA expression in control and treated whitefish ( $100\mu\text{g MC-LR}\cdot\text{kg}^{-1}$  body weight) exposed for 24 and 48h ( $n=3$ ). The bars represent mean values of expression ratios ( $\text{ER}\pm\text{S.E.}$ ), normalized by *rpl19*. Data were analyzed using randomization procedure (REST© 2008).

anti apoptosis (Chan et al. 2005). On the other hand, the abundant *omy-miR-125b* ( $\log\text{ER}=7.51$ ; S.E. 7.18-8.12;  $P<0.05$ ), which is a brain specific miRNA in fish and accounts for 14% of all clones isolated from rainbow trout miRNA library (Salem et al. 2009), was proven to be negative modulator of cellular tumor antigen p53 in both zebrafish and humans (Le et al. 2009).

Four miRNAs, *omy-miR-21t*, *omy-miR-199-5a*, *omy-miR-125a*, and *omy-miR-125t*, were represented in whitefish liver at lower, but comparable to one another, quantities (mean  $\log\text{ERs}$  from 6.19 to 6.81;  $P>0.05$ ). The „t” miRNAs that were, so far, only observed in the rainbow trout, *omy-miR-21t* and *omy-miR-125t* are of special interest because of their unique sequences and possibly unique targeting mechanisms

(Ramachandra et al. 2008). These miRNAs differ by at least one nucleotide and this difference might have profound impact on target recognition and post-transcriptional regulation. Thus, the differential liver expression of *omy-miR-125b* and *omy-miR-125t*, a miRNA specific to rainbow trout and possibly to other salmonids, suggests a complexity in post-transcriptional regulation by these miRNAs.

The remaining miRNA *let-7a* was the least abundant one among the miRNAs analyzed in the study (Figure 3). The relative low expression level of *omy-let-7a* observed in whitefish liver ( $\log\text{ER}=3.71$ ; SE 3.23-4.37;  $P<0.05$ ) may be consistent with its role in the regulation of cell growth and differentiation. For example, the *let-7* miRNA family regulates the *Ras* oncogenes; the expression of *let-7* inversely



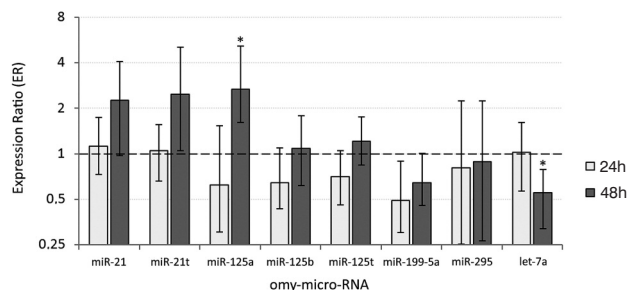
**Figure 3.** Expression pattern of whitefish miRNAs in liver of control fish. Quantity of each miRNA (logER) was normalized to omy-miR-295 (dashed line). Different letters denote mean miRNA quantities that are significantly different for the respective miRNA expression using ANOVA followed by Tukey's multiple comparison test ( $P < 0.05$ ).

correlates with expression of RAS protein in lung cancer tissues, suggesting a possible causal relationship in tumorigenesis (Johnson et al. 2005).

#### miRNA expression in whitefish treated with MC-LR

Except for two miRNAs, omy-miR-125a and omy-let-7a, the MC-LR treatment had no effect on the miRNAs examined in the study (Figure 4). Omy-miR-125a, that shows prominent expression in trout brain (Ramachandra et al. 2008; Salem et al. 2009), was up-regulated after 48h of the treatment (ER=2.68; S.E. 1.61-6.78;  $P < 0.05$ ) when compared to control. In rainbow trout individual development omy-miR-125a starts with a relatively high expression in unfertilized egg (0-2 dpf; *days post fertilization*), reaching a very low level in 2-dpf embryos and remains at low levels thereafter (Ramachandra et al. 2008). Herrera et al. (2009) found that increased miR-125a observed in liver of hyperglycaemic rats may particularly affect genes involved in the mitogen-activated protein kinases (MAPKs) signalling pathway. On the other hand it is known from studies with human cell lines that microcystin-LR is capable of activating several MAPKs, including ERK1/2, JNK, and p38 through inhibition of PP2A (Komatsu et al. 2007). If the two observations in mammals apply to fish, miR-125a may play a prominent role in liver cell responses to MC-LR.

The other altered miRNA, omy-let-7a, was down-regulated in whitefish liver after 48h of the treatment with MC-LR (ER=0.55; S.E. 0.32-0.79;  $P < 0.05$ ). Although alterations in let-7 miRNA expression have been thoroughly described in human cancer and may contribute to other disease states, it remains unclear what is driving such alterations in fish or if exposures to dangerous chemicals or conditions known to contribute to toxicity and diseases can modulate miRNA expression. Previous studies indicate that down-regulation of let-7 is a key event in lung cancer (Johnson et al. 2005). Considering multiple targets for a given miRNA, the regulatory pathways controlled by let-7 in the cancer disease may be involved in a very complex network. Indeed, besides the *Ras*,



**Figure 4.** Changes in miRNA expression in control and treated whitefish ( $100\mu\text{g MC-LR}\cdot\text{kg}^{-1}$  body weight) exposed for 24 and 48h ( $n=3$ ). The bars represent mean values of expression ratios (ER $\pm$ S.E.), normalized by omy-miR-122 as an endogenous reference, relative to a control sample (ER=1.00; dashed line) of a respective time of the experiment. Data were analyzed using randomization procedure (REST© 2008). Asterisks indicate groups that showed significantly higher levels of either mRNA over a respective control group ( $P < 0.05$ ).

a succession of oncogenes, including, c-Myc (Koscianska et al. 2007), NF2 (Meng et al. 2007), and several cell cycle genes (CDK6, CDC25A, and CCND2) (Johnson et al. 2007) have been shown to be modulated directly by the let-7 miRNA. Other important genes involved in the control of cellular proliferation, such as p21<sup>Waf1</sup>, may be regulated through let-7a-dependent negative modulation of the proteins' inhibitors (He et al. 2009). Therefore it is likely, that MC-LR that decreased expression of let-7a in the liver, had an effect in an enhancement or reduction of some, yet unknown genes that are involved in cell physiologic pathways in fish. Our attempts to discover let-7 function in fish in the context of MC-LR toxicity have just started. Further computational and experimental research is required, for prediction and verification of let-7a targets (genes) in fish. And more work with the fish is essential for understanding the crosstalk of the regulatory network controlled by let-7a, and other miRNAs.

We have shown previously (Brzuzan et al. 2009) that intraperitoneal injection of whitefish, *Coregonus lavaretus*, with MC-LR at subacute dose of  $100\mu\text{g}\cdot\text{kg}^{-1}$  body weight induced mRNA expression of tumor suppressor p53 and cyclin dependent kinase inhibitor 1 (cdkn1a) in the liver of exposed fish. Interestingly, the elevated transcript levels of both genes were observed only from 48 through the 72h of exposure. These observations have led us to a speculation that MC-LR may promote cell cycle arrest in the liver cells of whitefish through the action of cdkn1a (Brzuzan et al. 2009). However, the mechanism by which the apparent accumulation of mRNA was accomplished in the MC-LR stressed fish remains unknown. A new investigation is currently under way to study the regulatory network controlled by miRNAs in the context of MC-LR toxicity. We test, through computational prediction algorithms of whitefish p53 and cdkn1a genes, if miRNAs might be involved in their post-transcriptional regulation. From an environmental biotechnology perspective, the assay

presented here to study miRNA expression levels in whitefish exposed to microcystin-LR could be a potential technical tool for detecting the consequences of exposure of organisms to other environmental toxins.

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