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Shartau, Ryan B.; Snyman, Heindrich N.; Turcotte, Lenora; McCarron, Pearse; Bradshaw, Julia C.; Johnson, Stewart C.

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Acute microcystin exposure induces reversible histopathological changes in Chinook Salmon (*Oncorhynchus tshawytscha*) and Atlantic Salmon (*Salmo salar*)

Short running title: Acute microcystin exposure in salmon

Ryan B. Shartau[1*], Heindrich N. Snyman[2], Lenora Turcotte[1], Pearse McCarron[3], Julia C. Bradshaw[1], Stewart C. Johnson[1]

[1] Pacific Biological Station, Fisheries and Oceans Canada, Nanaimo, BC, Canada
[2] Animal Health Laboratory, University of Guelph, Kemptville, ON, Canada
[3] Biotoxin Metrology, National Research Council Canada, Halifax, NS, Canada.

*Current address: Department of Biology, The University of Texas at Tyler, Tyler, TX, United States

Author responsible for correspondence:
Ryan B. Shartau
Department of Biology
The University of Texas at Tyler
3900 University Blvd.
Tyler, TX, United States
75701
+1 (903) 508-0272
rshartau@uttyler.edu

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ABSTRACT

Atlantic Salmon (*Salmo salar*) and Chinook Salmon (*Oncorhynchus tshawytscha*) develop a severe liver disease called net-pen liver disease (NPLD), which is characterized by hepatic lesions that include megalocytosis and loss of gross liver structure. Based on studies where salmonids have been exposed to microcystin (MC) via intraperitoneal injection, NPLD is believed to be caused by MC exposure, a hepatotoxin produced by cyanobacteria. Despite the link between MC and NPLD, it remains uncertain if environmentally-relevant MC exposure is responsible for NPLD. To determine if we could produce histopathology consistent with NPLD, we compared the response of Atlantic and Chinook Salmon to environmentally-relevant MC exposure. Salmon were orally gavaged with saline or MC and sampled

over 2-weeks post-exposure. Liver lesions appeared by 6 h but were resolved 2-weeks post-exposure; histopathological changes observed in other tissues were not as widespread, nor was their severity as great as those in the liver. There was no evidence for NPLD due to the absence of hepatic megalocytosis. These results indicate that development of NPLD is not due to acute MC exposure but may be associated with higher MC concentration occurring in food, long-term exposure through drinking of contaminated seawater, and/or interactions with other marine toxins.

Key words
Atlantic Salmon (*Salmo salar*), Chinook Salmon (*Oncorhynchus tshawytscha*), *Microcystis aeruginosa*, Microcystin, Cyanobacteria, Net-pen liver disease, Hepatotoxin

INTRODUCTION

Microcystins (MCs) are cyanotoxins that are commonly identified in freshwater and less frequently from estuarine and marine waters (Preece et al., 2017). Species of cyanobacteria in the genus *Microcystis* are the most common bloom forming and considered to be the main producers of MCs in freshwater ecosystems (Preece et al., 2017). Sources of MCs in seawater are less well understood but include discharge from freshwater systems (Lehman et al., 2005; Miller et al., 2010; Preece, Moore, & Hardy, 2015; Robson & Hamilton, 2003), as well as production by brackish water and marine cyanobacteria belonging to numerous genera including *Anabaena*, *Anabaenopsis*, *Microcystis* and *Oscillatoria* (Miller et al., 2010; Preece et al., 2017; Robson & Hamilton, 2003; Tonk et al., 2007). Microcystins have also been detected in marine invertebrates such as mussels and crab larvae in northeast Pacific waters (Chen et al., 2016; Williams, Craig, et al., 1997).

Over 270 MC congeners have been identified (Pham & Utsumi, 2018), with MC-LR the most toxic and abundant, and well-studied followed, by MC-RR and MC-YR (Díez-Quijada et al., 2019; Pham & Utsumi, 2018). The toxicity of MC is largely due to the presence of the Adda residue (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) which inhibits protein phosphates leading to accumulation of phosphorylated proteins in the liver causing oxidative stress and cell necrosis (Malbrouck & Kestemont, 2006; Pham & Utsumi, 2018; Preece et al., 2017). Nodularins, structurally similar to MCs, are cyclic pentapeptides produced by cyanobacteria and also contain an Adda residue (Preece et al., 2017; Preece, Moore, Swanson, et al., 2015). Nodularins have similar hepatotoxic effects that are also mediated through their potent inhibition of protein phosphatases (Preece, Moore, Swanson, et al., 2015; Svirčev et al., 2015).

Microcystins are recognized as extremely potent hepatotoxins that are linked to changes in gene expression, physiology and morphology in numerous animals (Campos & Vasconcelos, 2010; Ma & Li, 2016; Woolbright et al., 2017) including fish (Malbrouck & Kestemont, 2006). In addition to causing severe liver damage and in some cases death, exposure to MCs can also result in a variety of sub-lethal effects in non-salmonids which involves cardiorespiratory function (Best et al., 2001; Martins et al., 2019), reproductive and endocrine function (Gao et al., 2020; G. Liu et al., 2017; Zhan et al., 2020), growth rate (Dong et al., 2012), whole animal condition (Acou et al., 2008), swimming performance (Cazenave et al., 2008), and immunity (Chen et al., 2016; Y. Li et al., 2009; Lin et al., 2017; W. Liu et al., 2014).

Phillips *et al.* (1985) produced the first detailed report of MC toxicity in fish by exposing Rainbow Trout (*Oncorhynchus mykiss*) to MCs via intraperitoneal (IP) injection of sonicated *Microcystis aeruginosa* cells. This exposure resulted in the rapid onset of massive necrosis within the liver, generalized loss of liver architecture and high rates of morbidity (Phillips et al., 1985). Shortly after this publication a severe toxicopathic liver disease, called Net-pen Liver Disease (NPLD) (Figure 1), was first reported from samples of seawater farmed Atlantic Salmon (*Salmo salar*) collected in 1986 in the northeastern Pacific in the Puget Sound of Washington State (Kent, 1990; Kent et al., 1988). This disease was characterized by loss of gross liver structure and histological changes that include diffuse necrosis, vacuolation and megalocytosis of the liver parenchyma (Kent, 1990) (Figure 2). These authors hypothesized that the responsible agent was a phycotoxin from algae to which the fish were exposed to by feeding on net pen fouling organisms or crab larvae (Kent et al., 1996; Williams, Craig, et al., 1997). In a follow-up to their 1990 study, liquid chromatography-linked protein phosphatase bioassay of liver extracts obtained from fish with NPLD demonstrated the presence of a protein phosphatase inhibitor that was chromatographically indistinguishable from MC-LR (Andersen et al., 1993). As liver tissue from

healthy fish showed an absence of MC-LR, Andersen et al. (1993) sought to replicate NPLD by IP injection of MC-LR into healthy Atlantic Salmon, which resulted in diffuse necrosis and hepatic megalocytosis; leading the authors to propose a linkage between MC-LR exposure and NPLD.

Since its first discovery NPLD, as identified by the presence of hepatic megalocytosis, has also been reported from farmed Steelhead Trout (*Oncorhynchus mykiss*), wild and farmed Chinook Salmon (*Oncorhynchus tshawytscha*), and wild Pink Salmon (*Oncorhynchus gorbuscha*) sampled in marine waters of the northeastern Pacific (Kent, 2015; Saksida et al., 2012; Stephen et al., 1993). In addition, there is evidence that the occurrence of NPLD on Atlantic Salmon farms in the northeastern Pacific has increased in frequency over the last several years (Kent, 2015). However, despite the importance of this disease to salmon production in the northeast Pacific it remains uncertain if MCs are indeed responsible for NPLD, and it is unknown how salmon would respond to environmentally relevant MCs exposure.

We compared the response of Atlantic and Chinook Salmon to environmentally relevant MC exposure to determine if we could produce histopathology consistent with NPLD, as well as to generated tissues for transcriptional studies and to support refinements to MC analysis methods. To this end post-smolt salmon were exposed by oral gavage to *M. aeruginosa* paste containing three different sub-lethal concentrations of MC, or saline under controlled laboratory conditions and sampled at 6, 12, 24, 72 hours, and 2-weeks post exposure. Differences between species with respect to the toxicity of MCs and the effects on liver pathology are reported in this study.

METHODOLOGY

Animal Care

All work with animals was performed in strict accordance with the recommendations in the Canadian Council on Animal Care (CCAC) Guide to the Care and Use of Experimental Animals. The protocols were approved by the Pacific Region Animal Care Committee (Animal Use Protocol Number: 18–008). All fish handling was performed under tricaine methanesulfonate (TMS; Syndel, Nanaimo, Canada) anesthesia.

Fish Sources and Husbandry

Atlantic and Chinook Salmon smolts were supplied by local commercial hatcheries in 2018 and transported to the Pacific Biological Station. Prior to transport fish were screened for disease agents and were found to be in good health. They were approximately 65 and 30 g upon arrival, respectively, and they were transitioned into seawater over a 2-week period. Fish were maintained in stock tanks in-flow through UV-treated seawater at ambient temperature (fluctuating between 8-13 ^{o}C) under a natural photoperiod. They were fed a commercial salmon feed at a rate of 1% body weight daily. Food was withheld 48 h prior to experiments.

Algae

The *Micocystis aeruginosa* culture CPCC-300 was obtained from the University of Toronto Culture Collection (now the Canadian Phytoplankton Culture Collection housed at the University of Waterloo, ON, Canada). A concentrated homogenous paste of CPCC-300 containing MC-LR and [Asp^3]MC-LR in approximately equal proportions (combined total of 110 μg g^{-1}) was produced at the National Research Council (NRC) (Halifax, NS) using bulk culturing conditions described previously for other strains of *M. aeruginosa* (update citation). The MC concentrations were verified using liquid chromatography-tandem mass spectroscopy (LC-MS/MS) at the NRC. In brief 0.2 g samples of biomass

were extracted in triplicate using 75% aqueous methanol with 0.1% acetic acid and made to a final volume of 50 mL. Filtered extracts were analysed using an Agilent 1290 LC system (Palo Alta, CA, USA) connected to a Sciex API 5500 QTRAP mass spectrometer (Concord, ON, Canada) with a Turbospray ionization source. Separations were on an Agilent Poroshell-120 SB-C18 column (150 × 2.1 mm, 2.7 µm), using a binary mobile phase of (A) H_2O and (B) MeCN/ H_2O (95:5), each with 50 mM formic acid and 2 mM ammonium formate. A gradient was run from 10 to 80% B over 30 min, and then re-equilibrated to 10% B before the next run. The flow rate was 0.25 mL min^{-1}, the column temperature was +30 °C, and the injection volume was 2.5 µL. Selected reaction monitoring conditions in positive ionization mode included a spray voltage of 5500 V and a source temperature of 375 °C. Primary SRM transitions for MC-LR (995.6 → 135.1) and [Asp^3]MC-LR (981.6 → 135.1) used a declustering potential of 75 V and a collision energy of 85 eV. Aliquots of *M. aeruginosa* paste were made by diluting paste with deionized water and was stored at -20 ^oC until used.

Experimental design and execution

Fish from each species were orally exposed to either saline (0.9% NaCl in deionized water containing food coloring) or *M. aeruginosa* at three MC concentrations – 1700 ("low"), 2200 ("medium"), or 3200 ("high") µg kg^{-1} fish body weight (based on an average weight of 75 g and 90 g for Atlantic and Chinook Salmon, respectively). Microcystin concentrations were chosen to be sublethal based on preliminary trials and data for rainbow trout (Tencalla et al., 1994). The saline and *M. aeruginosa* exposure groups were sampled at 6, 12, 24, 72 h, and 2-weeks post-exposure. Atlantic and Chinook Salmon were subjected to the same experimental protocol and held under the same conditions.

Prior to gavage, fish were randomly selected from the stock tank and placed in a bucket containing aerated seawater, and lightly anaesthetized with 0.02 g/L TMS. Fish were then placed in a V-shaped trough sprayed with Vidalife (Syndel, Nanaimo, Canada) to protect the mucous layer, then quickly gavaged using a 10 mL syringe with PE240 tubing that was marked to indicate approximate location of stomach. The tube had a blunted end to avoid internal abrasions, and was secured around a 1.5 inch 18 G needle.

After dosing, the tube was held in place for approximately 3-4 seconds to minimize regurgitation then gently withdrawn. The entire gavage procedure took <30 seconds per fish after which they were placed in a recovery bath, supported at approximately a 45-degree angle for about 2-3 minutes to prevent regurgitation. Fish were held in the recovery bath for about 10 minutes and observed for regurgitation, which if occurred was evident by discoloration of water. Fish which regurgitated the treatment were excluded from the study.

After recovery fish were placed in triplicate tanks for each treatment/species and held in sand filtered UV-treated 11°C seawater (32 ppt) for the 2-week duration of the experiment. Clinical signs of disease, mortality, water temperature and salinity were recorded daily.

Sampling and histological processing

Samples from seven fish of each species were taken at 6, 12, 24, 72 h, and 2-weeks post-exposure. At sampling fishes were euthanized (TMS 0.05 g L^{-1}) and lengths and wet weights obtained. Tissue samples for chemical analysis, transcriptional studies, and histology were collected in the following order: blood, gill (second right gill arch), liver, anterior and mid kidney, intestine, and brain. Tissues for histology (liver, mid kidney, intestine, and gill) were placed directly into histology cassettes, and were then fixed in 10% neutral buffered formalin for 48 h, then transferred to isopropanol for storage until processing.

At processing tissues were further dissected, embedded in paraffin, and sectioned at 4 µm. Sections were mounted on silane-treated glass slides and stained with hematoxylin and eosin (HE).

Histopathological changes were evaluated using a semi-quantitative scoring system which classifies lesion as follows: 0 = none, lesion is not present; 1 = mild, lesion/s encompass less than a 40X high power field or is < 0.5 mm in total size; 2 = moderate, lesion/s encompass > one 40X high power field but < than a 20X high power field or lesions are > 0.5 mm but less than 2.0 mm in total size; 3 = severe, lesion/s encompass > than a 20X high power field or lesions are > 2.0 mm in total size (Wolf & Wolfe, 2005).

Statistical analysis

Histological scores across treatments were compared using an ANOVA based permutation test, a two-way permutation t-test was used to compare lesion scores between Atlantic and Chinook Salmon at the same sampling points. GraphPad Prism (v.8) and R version 3.6.2 (The R Foundation for Statistical Computing) was used for statistical analyses and preparation of figures.

RESULTS

Overview

Atlantic and Chinook Salmon did not experience mortality during MC exposure but behavioural changes were observed over the first 8-10 days, and reduced feeding in the high MC group persisted over the 2-week experimental period. There were three primary histopathological features observed in the liver following gavage – hepatocellular hydropic degeneration (HHD), basophilic cytoplasm (BPC), and cytoplasmic vacuoles (VAC). In both species HHD was observed at 6 h and resolved by 2-weeks. Only Atlantic Salmon were observed to have BPC which was largely resolved by 2-weeks. Chinook and Atlantic Salmon were found to exhibit VAC in all treatment groups, including saline, being most severe at 2-weeks. In other tissues, there were few histopathological features, which largely consisted of infrequent and minor incidences of gill lamellar branchitis (GLB) in both species.

Fish appearance, behaviour, and mortality

Behavioral changes and other signs of morbidity were observed over the 2-week sampling period. Following gavage both species displayed very little interest in food over the first 48 h in all treatments after which, most fish started feeding with saline treatment fish displaying near normal feeding behaviour after 72 h. In contrast it took 8 – 10 days for fish in the low and medium MC treatment groups to display near normal feeding behaviour; with feeding in the high MC group never recovering fully over 2-weeks. Swimming behaviour and startle response were visibly reduced following gavage with recovery of these behaviours mirroring feeding behaviour.

The levels of MC used in this study were selected to cause physiological and pathological changes from which the fish could recover without causing mortalities. There are few data on the oral toxicity of MCs in fish and our selection of doses was based on experiments on Rainbow Trout which reported no mortality following a single oral gavage with 1700 µg MC-LR kg^{-1} body weight at 96 h (Tencalla et al. 1994), and 5700 µg MC-LR kg^{-1} body weight at 72 h (Tencalla & Dietrich, 1997), but 100% mortality within 96 h at 6600 µg MC-LR kg^{-1} body weight (Tencalla et al., 1994). Tencalla et al. (1994) also reported that Rainbow Trout which received 4400 µg MC-LR kg^{-1} body weight over 8 doses displayed signs of severe toxicity over 96 h. In all but the lowest dose (1700 µg MC-LR kg^{-1} body weight) in

which behavioral and histological changes were not observed, single doses of MC at these levels resulted in signs of acute toxicity with severe gross and histological signs of liver damage.

Histopathological changes in the liver
In the liver there were three primary histopathological features observed following gavage – hepatocellular hydropic degeneration (HHD), basophilic cytoplasm (BPC), and cytoplasmic vacuoles (VAC) (Table 1 and 2).

Hepatocellular hydropic degeneration is defined as the swelling of the hepatocytes with loss of cytoplasm staining intensity and characteristic foamy cytoplasmic vacuolation. In Atlantic Salmon, treatment and time significantly affected HHD score ($Pr<0.001$). HHD features were most severe in the medium and high treatment groups at 6 h with mean lesion score being 2.3 and 2.2 (Figure 3), respectively. At 6 h the scores for the medium and high features differed from saline treatment ($p<0.05$), at 12 h the scores differed between the saline and medium ($p<0.01$), low and high ($p<0.05$), medium and high ($p<0.01$) groups; no differences between treatment groups were observed at 24, 72 h or 2-week time points.

The HHD features in Chinook Salmon were less severe but they were significantly affected by treatment ($Pr<0.001$) and time ($Pr<0.001$); at 72 h the high treatment histopathological features differed from the saline group ($p<0.05$). In both species, incidences of HHD were resolved by 2-weeks with no evidence of this histopathological feature in any of the fish which were examined (Figure 3).

Basophilic cytoplasm (BPC) is a histopathological feature that is characterized by the reaction of cytoplasmic proteins and RNA with eosin to increase staining intensity (Ross & Pawlina, 2006). Atlantic Salmon, but not Chinook Salmon, developed BPC which were affected by treatment ($Pr<0.01$), time ($Pr<0.01$), and the interaction of time and treatment ($Pr<0.01$). The presence of BPC was most severe at 12 h in the high treatment (Figure 4). Similar to the HHD, BPC appeared to be fully resolved by 2-weeks.

The third prominent histopathological feature that was observed presence of vacuoles in the cytoplasm (VAC) of hepatocytes. With the exception of Chinook Salmon at 6 and 12 h, and Atlantic Salmon at 2-weeks, VAC were generally more common in the saline group when compared to all of the MC treatment groups at all time points in both (Figure 5). The occurrence of this histopathological feature in Atlantic Salmon in all of the treatment groups was generally reduced from 6 to 72 h when compared to the saline control group. In Chinook Salmon this reduction was first evident at 24 h with VAC scores being the lowest at 72 h. In general, Atlantic Salmon showed a significantly greater reduction in VAC when compared to Chinook Salmon (two sample permutation t-test, $P<0.05$.

Comparing scores of HHD, BPC, and VAC between Atlantic and Chinook Salmon at each time point for each treatment indicated that scores were not significantly different between the species. However, BPC was not observed in Chinook Salmon. Figures 6 and 7 show selected examples of these lesions.

Histopathological changes in other tissues
Histopathological changes observed in other tissues were not as widespread, nor was their severity as great as those in the liver (Table 1 and Table 2). In Chinook Salmon kidney, there was evidence of mineralization in nine fish, four of which were saline, all of which were scored ‘1’ and there were no differences between treatments or across time. In the gills, Chinook Salmon developed gill lamellar branchitis (GLB) with maximum score of ‘1’; there was a significant effect of time ($P<0.001$) and the

interaction between time and treatment ($P<0.05$). Several Atlantic Salmon also developed GLB with a maximum score of '1'; there were no significant differences. In the other tissues examined – intestine, heart, spleen, and ovaries – there were no histopathological changes observed in either Atlantic or Chinook Salmon.

DISCUSSION

A key objective of this study was to investigate if acute exposure of microcystin containing cyanobacteria would reproduce the histopathology consistent with NPLD, specifically the development of hepatic megalocytosis. Our results demonstrated that NPLD did not develop as a result of the type of MC exposure used here, and that, not surprisingly, salmon respond differently to MC delivered in a cyanobacterial paste by gavage compared to previous reports of purified MC injected into the intra-peritoneum. While Atlantic and Chinook Salmon did not develop signs of NPLD, we show that orally consuming cyanobacteria containing microcystin in these concentrations rapidly produces hepatic lesions that are resolved within 2-weeks of exposure. Thus, this indicates that while microcystin exposure is acutely damaging, a single exposure does not induce NPLD and that a sustained subclinical/sub-lethal exposure may be required for the development of NPLD.

Andersen et al. (1993) injected salmon three times over 3 days with purified MC at a concentration of 555 μg kg^{-1} for a total MC exposure of 1660 μg kg^{-1} to reproduce the clinical signs of NPLD in Atlantic Salmon; however, while this study informs on the hepatotoxicity of purified MC in Atlantic Salmon, it is understood that the route of exposure and use of purified toxin instead of algae produces vastly different outcomes in fish. Tencalla and colleagues (Tencalla et al., 1994) demonstrated these differences in Rainbow Trout comparing purified toxin and freeze-dried algae containing MC administrated by IP injection, gavage and water-borne exposure. A single gavage of freeze-dried algae containing MC at 6600 μg kg^{-1} resulted in severe liver damage in trout and was fatal within 72 – 96 h; gavage with 1700 μg kg^{-1} did not result in liver damage or mortality (Tencalla et al., 1994). These results differed from treatment with purified toxin during the gavage trial as gavage of 1200 μg kg^{-1} did not produce morbidity but IP injection with same MC concentration as freeze-dried algae resulted in mortality within 24 h. Repeated gavage in trout over four days with 8 exposures of 550 μg kg^{-1} produced modest to severe liver damage but no mortality prior to sampling at 96 h and the hepatic tissue had inflammatory response and focal necrosis. These results from the gavage trials are in sharp contrast with IP injection of freeze-dried algae at 550 μg kg^{-1} which resulted in severe hepatic necrosis and mortality within 24 h (Tencalla et al., 1994); a similar response was also observed when Atlantic Salmon IP injected with purified toxin in a pilot study (H. Snyman, personal observations). When Carp (*Cyprinus carpio*) were given single IP injection of 25 and 50 μg kg^{-1} purified MC resulted in mortality at 48 and 5 hours, respectively; this differed from when carp were gavaged as they only experienced morbidity at 250 μg kg^{-1} where megalocytosis was occasionally observed in the liver (Carbis et al., 1996). In Channel Catfish (*Ictalurus punctatus*) IP injected with purified MC at a concentration of 1900 μg kg^{-1} was lethal but gavage with a maximum MC concentration of 20,000 μg kg^{-1} was not lethal (Snyder et al., 2002). Taken together these studies emphasize the importance of exposure route and whether MCs are delivered as a purified toxin or in association with algal cells in MC toxicity. Exposure of fish to toxic algal cells makes it difficult to fully distinguish the potential microcystin effects from those caused by other components of the cells, and may explain some of the variability between studies. *Daphina galeata* exposed to toxic *M. aer*uginosa experienced feeding reductions, however, these were associated with the non-microcystin cellular components (Rohrlack et al., 1999). Additionally, algal cells produce a mixture

of isoforms which vary between *M. aeruginosa* strains which could introduce additional variation in the responses (Pham & Utsumi, 2018).

Microcystins are large molecules that are unable to passively penetrate through cell membranes and thus require active transport. Transport of MC has been found to occur via organic anion transporting polypeptides (OATPs) in zebrafish (Faltermann et al., 2016; Popovic et al., 2010; Steiner et al., 2016), trout (Steiner et al., 2014), and skate (Meier-Abt et al., 2007), as well as mammals (Feurstein et al., 2009; A. Fischer et al., 2010; McLellan & Manderville, 2017). In order for MC to produce their toxic effect, they must be taken up and transported into the body. Microcystins administered by gavage will be limited by the capacity for transport from the digestive tract into the body unlike IP injection; the gastrointestinal tract is the primary site of MC absorption but absorption may differ between fish species due to differences in gastrointestinal characteristics, as a longer intestine may provide a greater surface area for toxin absorption (Bury et al., 1998). This study and others do not indicate what percentage of MC is taken up by gavage relative to IP injection as only a portion of MC is taken up across the digestive tract. Additionally, MC delivered by gavage will enter the body cavity by way of the hepatic portal vein which is the typical route for nutrient and toxin absorption compared to MC arriving by IP injection which is freely available in the peritoneum. As gavage represents a more physiologically relevant route of exposure, fish are likely to have a greater capacity to deal with toxins arriving this route compared to them freely entering the peritoneal cavity.

Although the hepatic lesions observed in this study differ from those arising from IP injection by Andersen et al. (Andersen et al., 1993), they are consistent with the hepatotoxic effect arising from MC exposure. Gavaged Atlantic and Chinook Salmon developed hydropic degeneration (HHD) lesions within the first 72 h of MC exposure, this lesion was also observed in IP injected Atlantic Salmon, although the severity was likely greater as diffuse necrosis and hepatocyte hypertrophy occurred as well (Andersen et al., 1993). HHD is a reversible response to acute toxicity that results in a disruption to hepatocyte ion and fluid homeostasis leading to an increase in intracellular water (Abdelhalim & Jarrar, 2011); prolonged or repeated toxin exposure will prevent recovery and lead to necrosis (Bischoff et al., 2018). In addition to the HHD lesion, Atlantic Salmon exposed to MC developed BPC lesions which were most prominent at the high MC dose; the presence of basophilic cytoplasm is caused by the presence of increased amounts of RNA in the cytoplasm (Ross & Pawlina, 2006) and can be associated with inflammation and increased cellular metabolism (Harvey, 2012; Samour et al., 2016); Chinook Salmon did not develop BPC lesions. Similarly, the HHD lesions in Chinook Salmon in the MC exposed fish were less severe than in Atlantic Salmon (two sample permutation t-test, $P<0.05$); that Chinook Salmon lesion severity was lower than in Atlantic Salmon suggests that they are more tolerant of MC exposure. While the overall differences in lesion severity is small, differences between Chinook and Atlantic Salmon may be due to the environmental conditions in which they have evolved; it remains unknown whether natural MC exposure differ between the two species.

MC concentrations can vary substantially depending on local conditions that create blooms in marine and freshwater environments, MC concentration is also greatly influenced by freshwater discharge which elevated MC concentrations in estuaries (Preece et al., 2017; Preece, Moore, & Hardy, 2015). Based on existing information, the west coast of North America where Chinook Salmon are found may experience higher MC concentrations than the Atlantic Ocean, this may be due to a combination of harmful algae blooms and freshwater run-off, however, this information is overall limited to a few specific regions so a proper comparison is not possible with the current data. Around southern Vancouver Island in British Columbia Canada, dissolved MC concentration in marine waters range from 0.4-1435 $\mu g\ L^{-1}$ (Shartau et al., unpublished observations) which is higher than values measured in

estuaries in the Atlantic (Wood et al., 2014) and comparable to concentrations found in California (Gibble & Kudela, 2014; Peacock et al., 2018).

The other hepatic lesion that developed in Atlantic and Chinook Salmon was vacuolation of hepatocyte cytoplasm (VAC); these lesions were not specifically associated with MC exposure. Vacuolization of hepatocytes is a sign of hepatic steatosis and can arise in response to a number of issues (Spisni et al., 1998). Here, the development of vacuoles in the hepatocytes indicates that the fish are experiencing distress (Spisni et al., 1998), which may be due to nutrition and/or stress of gavage and subsequent isolation in tanks. As fish never resumed normal eating behavior and this change could have altered metabolism and induced stress. Additionally, due to space limitations, there were 3-4 per treatment tank which resulted in low fish density but low density also can lead to the development of social hierarchies within tanks where certain individuals are dominant and submissive in Rainbow Trout (Mussa & Gilmour, 2012). As these social hierarchies can lead to individual experiencing stress creating behavioural and physiological changes in Rainbow Trout (Culbert & Gilmour, 2016) that include changes to lipid metabolism (Kostyniuk et al., 2018), it could be expected that this would occur in other salmonids (X. Li et al., 2003; Trowell, 1946), thus the physiological significance of these vacuoles should be interpreted with caution.

No lesions were observed in other tissues with the exception of gill lamellar bronchitis (GLB) in Chinook Salmon where a total of 39 fish had minor GLB lesions. The presence of GLB suggest minor branchial inflammation in Chinook Salmon. As mean lesion score was highest at 6 h and 2-weeks post-gavage, it suggests that initially the branchial irritation could be associated with the gavage procedure; it is uncertain as to why Chinook Salmon more susceptible to these lesions than Atlantic Salmon where it only appeared 3 individuals throughout the study. In Chinook Salmon several fish developed mineralization in kidneys but as there was no significance effect of treatment or time, it is unlikely mineralization was related to experimental trials.

In the other tissues (heart, intestine, ovaries, and spleen) examined no lesions were observed, indicating neither gavage nor MC exposure induced any gross morphological effects. In Carp exposed to MC exposure via gavage reported histological changes to liver (Carbis et al., 1996), renal proximal tubular cells and intestinal mucosa (Fischer & Dietrich, 2000), and Medaka gavaged with MC had cellular changes with hepatocyte cytoplasm containing small bubble-like structures (suggestive of hydropic degeneration), dense nuclear chromatin, and some exhibited breakdown of the nuclear membrane, mitosis arrest, and dilation of endoplasmic reticulum (Qiao et al., 2019). Dietary consumption of MC by Threadfin Shad (*Dorosoma petenense*) induced histological changes to liver and the female gonads (Acuña et al., 2012) and Tenca (*Tinca tinca*) developed changes to hepatocytes, glomerulopathy in the kidney, loss of myofibrils in the heart and vacuolated enterocytes in the gastrointestinal tract (Atencio et al., 2008). Liver was the only organ experiencing histological changes in Channel Catfish exposed to MC via gavage, IP injection, and immersion (Snyder et al., 2002), while MC exposure via IP injection in Rainbow Trout resulted in kidney lesions consisting of coagulative tubular necrosis with a dilation of Bowman's space (Kotak et al., 1996). This indicates that tissue specific histopathological changes may be species and exposure route specific, however many of the effects of MC exposure are not visible as biochemical and gene expression changes are commonly observed. In addition to the above histopathological changes induced by MC exposure, there are a range of physiological and behavioral effects that arise throughout the life stages of numerous fish species (reviewed by Malbrouck & Kestemont, 2006).

Atlantic and Chinook Salmon may naturally encounter MC in the marine environment as MC has been documented to occur in marine and estuaries worldwide (Preece et al., 2017), including in coastal British Columbia where wild and farmed Chinook Salmon and farmed Atlantic Salmon are present (Shartau et al., unpublished). While the concentrations of MC in marine environments are generally lower than those in freshwater (Preece et al., 2017), salmon may be acutely exposed to high levels due to localized factors due to blooms (Paerl & Otten, 2013) and chronically exposed as MC appears to be persistent year-round (Peacock et al., 2018; Shartau et al., unpublished). The primary route of MC uptake by salmon is not known but is likely to be drinking or dietary consumption. Absorption across the epithelial surface of the gills and skin is unlikely to be significantly toxic as fish immersed in MC containing water do not generally experience toxic effects (Carbis et al., 1996; Snyder et al., 2002; Tencalla et al., 1994), which may be a consequence of the lack of transporters (i.e. OATPs) to facilitate MC uptake at these sites (McLellan & Manderville, 2017; Steiner et al., 2016).

As marine fishes, including seawater acclimated salmon, drink to maintain osmoregulatory balance, this is a potential source of MC uptake. Drinking rates in seawater acclimated Rainbow Trout (Shehadeh & Gordon, 1969), Coho Salmon (Damsgaard et al., 2020), and Atlantic Salmon (Fuentes & Eddy, 1997; Usher et al., 1988) have been measured to be up to 129, 288, and 192 mL h^{-1} kg^{-1}, respectively. Based on the upper concentration of seawater MC concentration found in British Columbia (1435 μg L^{-1}; Shartau et al., unpublished), these fish could consume 185, 413, and 276 μg kg^{-1} day^{-1}, respectively, which greatly exceeds the chronic tolerable daily MC intake level of 40 μg kg^{-1} day^{-1} for human consumption recommended by the World Health Organization (Preece et al., 2017). Dietary consumption of MC may represent another important source of MC as they have been found to bioaccumulate in organisms native to salmon habitats such as Dungeness crab larvae (*Cancer magister*) (Williams, Craig, et al., 1997) and saltwater mussels (*Mytilus edulis*) (Williams et al., 1997) which have been found to have MC concentrations of up to 84 and 63 ug g wet $tissue^{-1}$, respectively. Despite these high MC concentrations, almost all MC was covalently bound with approximately 0.007 and 0.03%, respectively, being freely available; it is unknown if covalently bound MC is biologically available when larvae are consumed, and within the context of NPLD, whether covalently bound MC participates in the etiology of NPLD (Williams, Craig, et al., 1997). Bioaccumulation and biomagnification of MC by plants (Cao et al., 2019) and animals (Ger et al., 2018; Miller et al., 2010; Zhang et al., 2009) throughout food webs (Pham & Utsumi, 2018) is known to occur and can negatively impact fish health (Bi et al., 2019; Vasconcelos et al., 2013; Zamora-Barrios et al., 2019). Salmon are also likely impacted by the accumulation of MC from food sources; this should be investigated for possible links to histopathological changes observed in farmed and wild fish associated with MC exposure, and also related to the etiology of NPLD.

In summary, our study demonstrates that Atlantic and Chinook Salmon acutely exposed to MC by gavage experience severe but reversible lesions in the liver but does not result in NPLD. Within 6 h following MC exposure hepatic lesions were evident in both species but were fully resolved by 2-weeks post exposure indicating that these salmonids are sensitive to oral exposure of MC at concentrations ranging from 1700 to 3200 μg kg^{-1}, but these concentrations were not lethal, largely only affected the liver, and fish fully recovered. These data provide insight into the histopathology of severe acute ecologically relevant MC exposure and demonstrate that although Atlantic and Chinook Salmon are affected, they have the capacity to recover which suggests they are able to tolerate severe short-term MC exposure arising from dietary consumption and cyanobacterial blooms. Future work will investigate the molecular and biochemical changes in Atlantic and Chinook Salmon in response to these MC exposures which will provide a more comprehensive understanding of the consequences of MC on these species; additionally, we aim to investigate the role of chronic MC exposure in the etiology of NPLD. Studies investigating the response of salmon to MC is relevant to understanding how salmon are affected by

naturally occurring cyanotoxins which have the potential to impair a range of physiological and behavioral functions, ultimately affect individual and population survival, and play a role in the ecosystem level issues related to salmon, including aquaculture (Afonso, 2020; Sinden & Sinang, 2016).

FIGURES

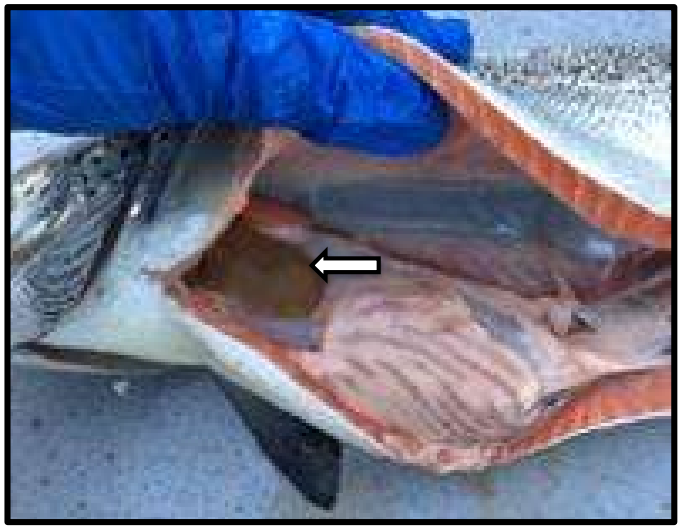

Figure 1: Liver displaying severe net-pen liver disease (NPLD) collected during an outbreak at a fish farm in British Columbia. Liver is indicated by the arrow; note the discoloration.

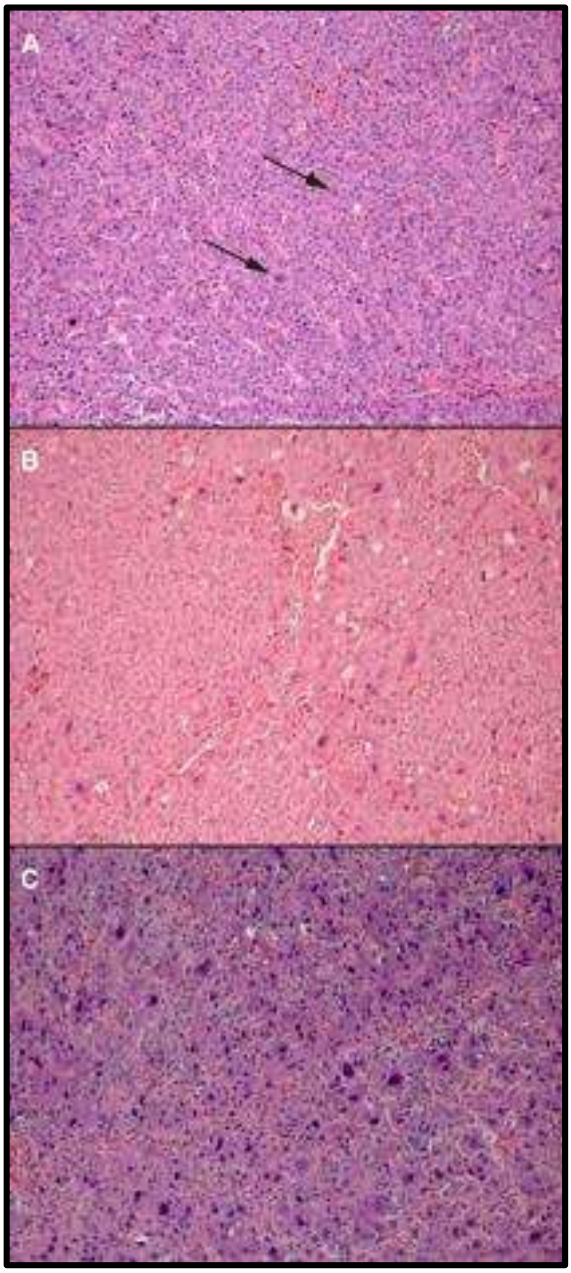

Figure 2: Examples of farmed Atlantic salmon displaying various stages of NPLD from 2017. (A) An example of a rating of plus one. Normal architecture still present in liver but the occasional megalocytosis present (black arrow). (B) An example of rating plus two. The liver is beginning to lose the normal architecture and megalocytosis is much more frequent in certain areas but there is also still a presence of normal architecture in other areas. (C) Example of rating plus three. Complete loss of normal architecture and numerous megalocytosis throughout liver.

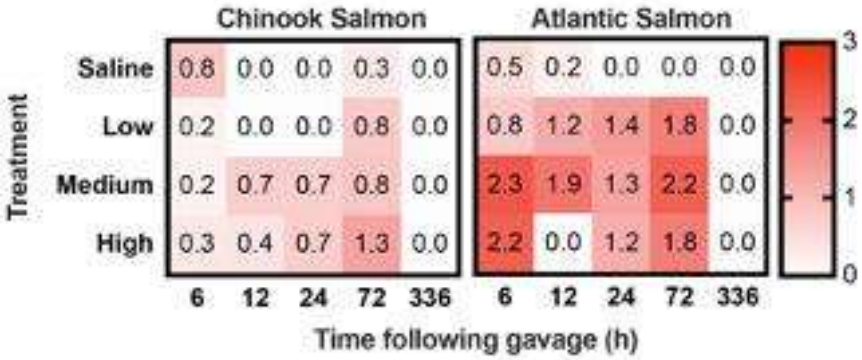

Figure 3: Heatmap displaying the mean score for hepatocellular hydropic degeneration (HHD) following gavage of saline or microcystin at low, medium, or high concentration in Chinook and Atlantic Salmon. Lesion scores are 0 = absent, 1= mild, 2 = moderate, 3 = severe; legend displays the color associated with scores in heatmap.

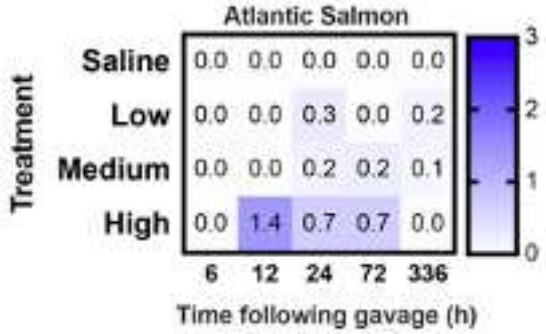

Figure 4: Heatmap displaying the mean score for basophilic cytoplasm in hepatocyte (BPC) following gavage of saline or microcystin at low, medium, or high concentration in Atlantic Salmon. This lesion did not appear in Chinook Salmon. Lesion scores are 0 = absent, 1= mild, 2 = moderate, 3 = severe; legend displays the color associated with scores in heatmap.

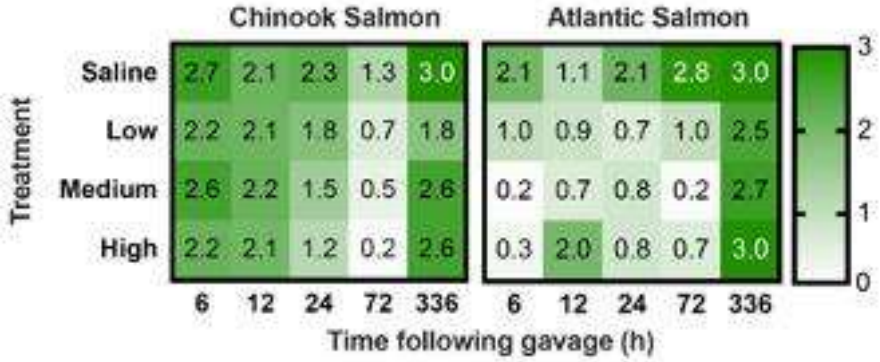

Figure 5: Heatmap displaying the mean score for vacuolation of hepatocyte cytoplasm (VAC) following gavage of saline or microcystin at low, medium, or high concentration in Chinook and Atlantic Salmon. Lesion scores are 0 = absent, 1= mild, 2 = moderate, 3 = severe; legend displays the color associated with scores in heatmap.

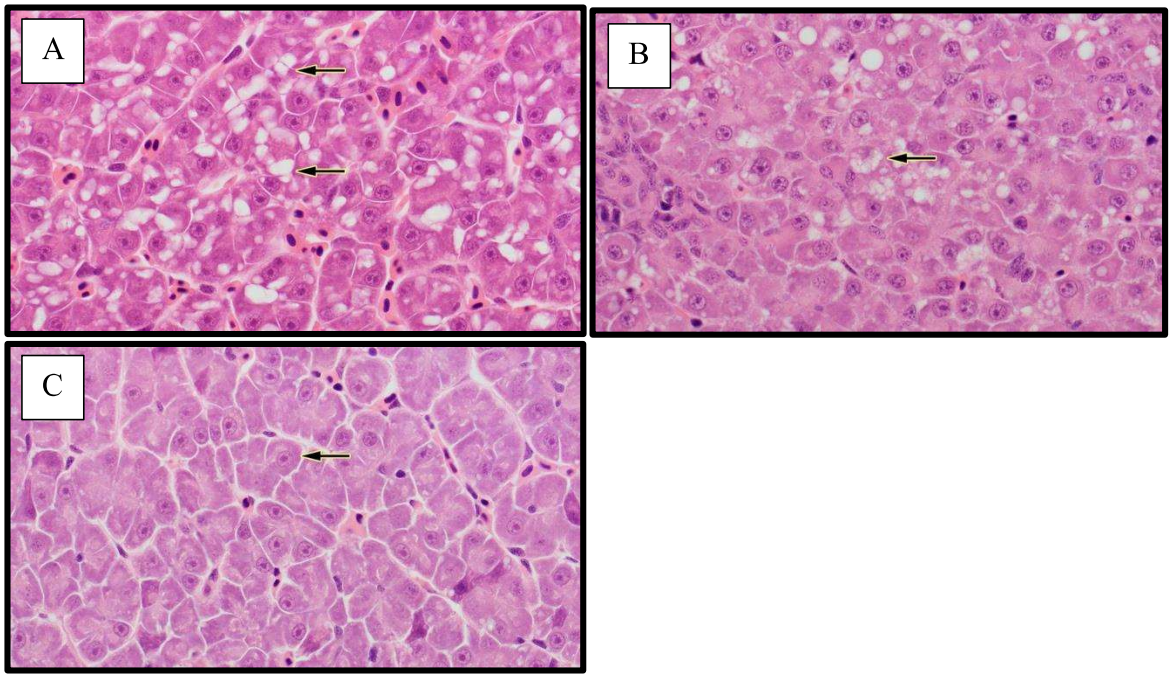

Figure 6: Selected examples of hepatic lesions in Atlantic Salmon. Severe vacuolation of hepatocyte cytoplasm (VAC) occurring 12 hour following gavage with saline (A). Severe hepatocellular hydropic degeneration (HHD) occurring 12 hours following gavage of high microcystin concentration (B). Mild basophilic cytoplasm (BPC) occurring 72 hours following gavage of high microcystin concentration (C). Arrows indicate example of lesion; staining with hematoxylin and eosin, 60x magnification.

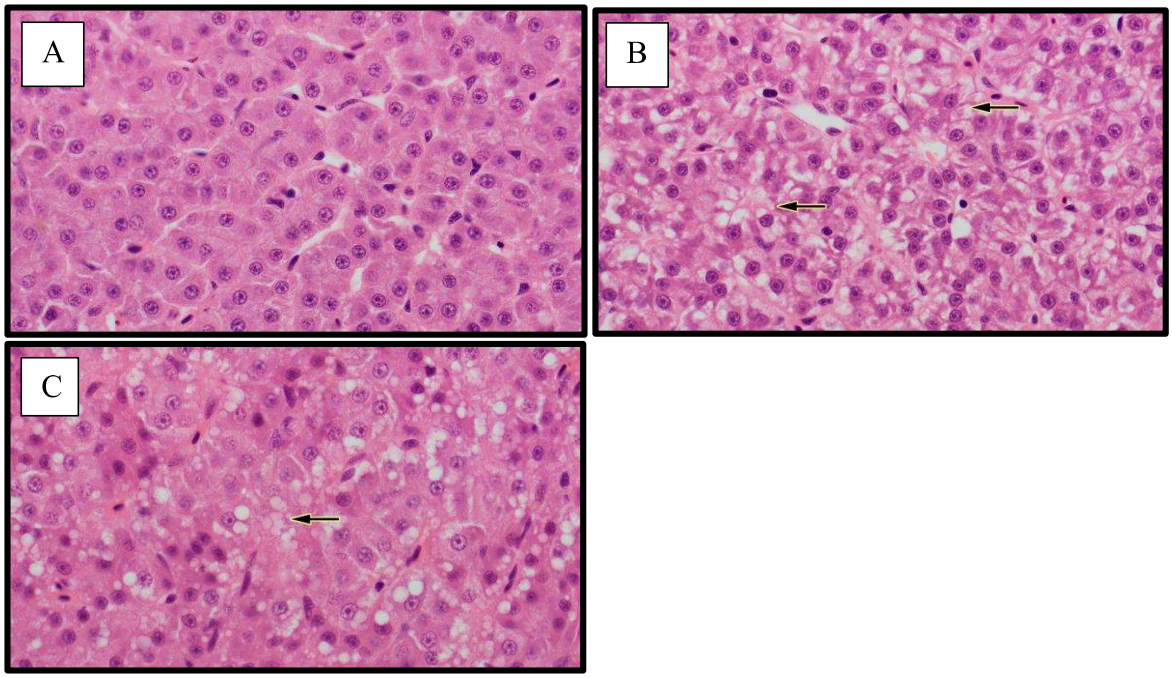

Figure 7: Selected examples of hepatic lesions in Chinook Salmon. At 24 hours post gavage with high microcystin concentration no significant lesions were observed (A). Severe vacuolation of hepatocyte cytoplasm (VAC) occurring 72 hours following gavage with saline (B). Moderate hepatocellular hydropic degeneration (HHD) occurring 72 hours following gavage with medium microcystin concentration. Arrows indicate example of lesion; staining with hematoxylin and eosin, 60x magnification.

Table 1 – Scoring of lesions in Atlantic Salmon liver, kidney, and gills following gavage of saline or microcystin at low, medium, or high concentration in Chinook and Atlantic Salmon. Lesion scores are 0 = absent, 1= mild, 2 = moderate, 3 = severe. Min and max indicate the minimum score and maximum score observed for that lesion, count is the total number of samples examined, and below indicates the number of samples with the lesion scores. Abbreviation are as follows: VAC – vacuolation of hepatocyte cytoplasm, BPH – biliary preductular cell hyperplasia, HHD – hepatocellular hydropic degeneration, MIN – mineralization, GLB – gill lamellar bronchitis.

	Liver				**Kidney**	**Gill**
	VAC	BPH	BPC	HHD	MIN	GLB
Min	0	0	0	0	0	0
Max	3	1	1	3	0	1
count	130	130	130	130	128	129
n = 0	55	128	115	71	128	126
n = 1	13	2	15	12	0	3
n = 2	17	0	0	20	0	0
n = 3	45	0	0	27	0	0
mean score	1.40	0.02	0.12	1.02	0	0.02

Table 2 – Scoring of lesions in Chinook Salmon liver, kidney, and gills following gavage of saline or microcystin at low, medium, or high concentration in Chinook and Atlantic Salmon. Lesion scores are 0 = absent, 1= mild, 2 = moderate, 3 = severe. Min and max indicate the minimum score and maximum score observed for that lesion, count is the total number of samples examined, and below indicates the number of samples with the lesion scores. Abbreviation are as follows: VAC – vacuolation of hepatocyte cytoplasm, BPH – biliary preductular cell hyperplasia, HHD – hepatocellular hydropic degeneration, MIN – mineralization, GLB – gill lamellar bronchitis.

	Liver				**Kidney**	**Gill**
	VAC	BPH	BPC	HHD	MIN	GLB
Min	0	0	0	0	0	0
Max	3	1	1	3	1	1
count	158	158	158	158	158	158
n = 0	23	155	156	125	149	119
n = 1	27	3	2	24	9	39
n = 2	44	0	0	8	0	0
n = 3	64	0	0	1	0	0
mean score	1.94	0.02	0.01	0.27	0.06	0.25

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