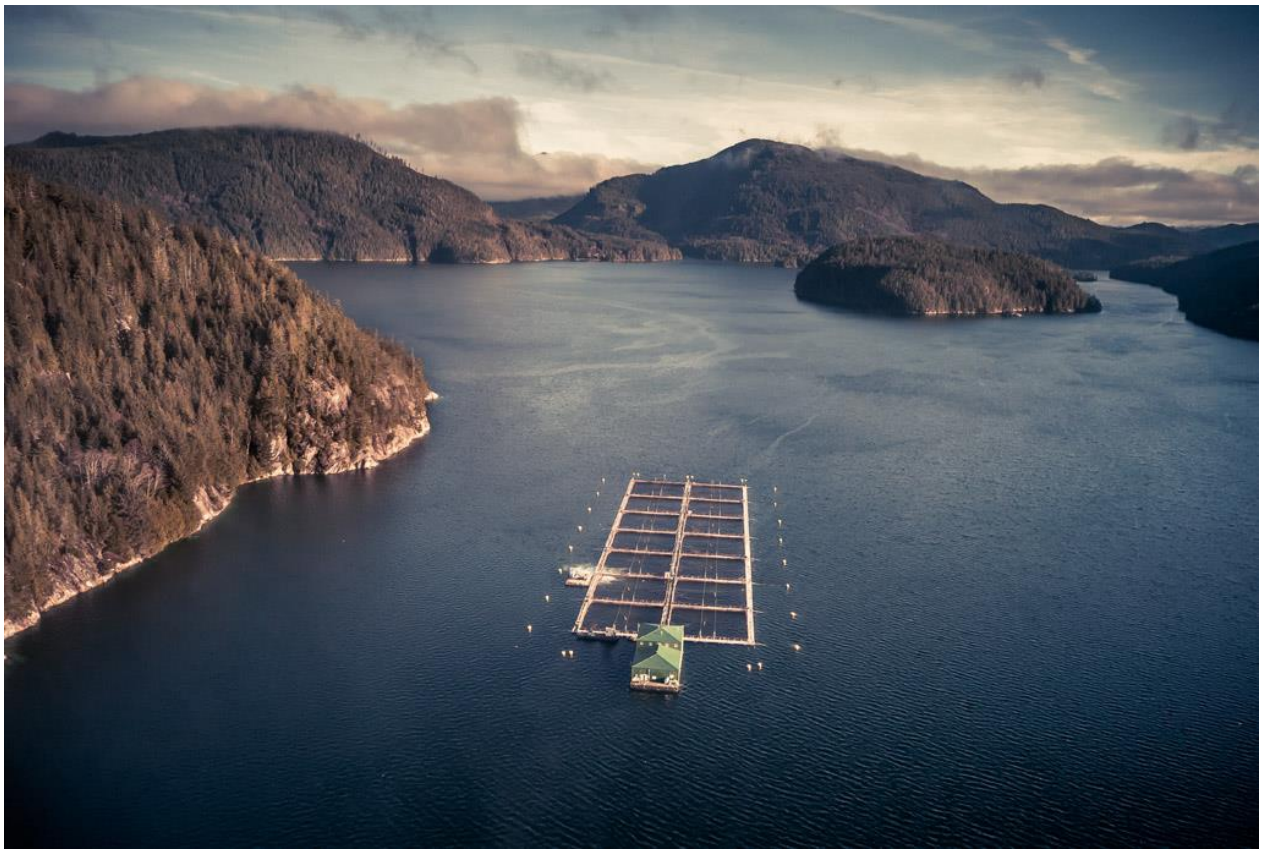
Report and Synopsis for the Workshop

Netpen Liver Disease: What we know. What we don't know.



Overview

The ocean is a dynamic environment. Needless to say recent changes in the oceanic systems demonstrates its nonstative nature. There is the presence of the "warm blob" off the coast of the Pacific North West. There is the acidification of the Pacific waters over the past several years affecting all shellfish. There is the formation of a massive algae bloom of Pseudo-nitzschia spanning from California to Alaska.

Like these variances in the marine environment, recent changes have also been observed in the epidemiology of Netpen Liver Disease. Traditionally observed in salmon newly introduced into the ocean, larger fish now living in the saltwater environment for well over a year have been diagnosed with the disease. The scenario of small fish grazing on the net pens fouled with toxin producing algae was speculated as the means of delivery of the causative agent for the disease. This premise however no longer applies as the older fish are well established onto feed.

Many questions accompany this shift. We know the specific pathology. We don't know specifically the causative agent. We don't know the means the causative agent is introduced into the fish. Even the origin of the causative agent is in question. Six presentations in this work shop span topics including the history of the disease, the most recent presentation of the disease, the pathology, possible origins, and possible connections with the freshwater ecosystem. Toba Beta stated “At beyond continent of reality, there are oceans of ideas”. Many ideas were generated from discussions inspired from the presentations. These ideas have been captured in the discussion section along with the research proposals that will hopefully provide pathways to answering these questions.

Contents

Attendance List

Full Name	Company	Job Title
Stephanie Smith	Beagle Bioproducts	CSO and COO
Nicky Haigh	HAMP, Microthalassia Consultants	Program Manager, CEO
Hein Snyman	Ministry of Agriculture, Animal Health Centre	Fish Pathologist
Michael Kent	OSU	Research Professor
Asit Mazumder	University of Victoria	Research Professor
Joanne Liutkus	BC Salmon Farmers Association	Research & Development Coordinator
Ahmed Siah	Centre for Aquatic Health Sciences	Research Scientist/Microbiology Diagnostician
Jim Powell	Centre for Aquatic Health Sciences	Chief Executive Officer
Tina Podlasly	Centre for Aquatic Health Sciences	
Tyler Stitt	Centre for Coastal Health	Core Researcher
Megan Sidey	Cermaq Canada	Fish Health Technician
Kathleen Frisch	Cermaq Canada	Fish Health Technical Manager
Nathan Cassan	Cermaq Canada	Fish Health Manager
Peter McKenzie	Cermaq Canada	Fish Health Director
Cecile Van Waensel	Elanco Animal Health	Accounts BC
Celine Leichner	Europharma	Accounts Manager BC
Paula Galloway	Ewos Canada Ltd.	Marketing Manager
Ian Keith	Fisheries and Oceans	A/Lead Veterinarian
Stewart Johnson	Fisheries and Oceans	Head Aquatic Animal Health
Marc Trudel	Fisheries and Oceans -	Research Scientist
Barry Milligan	Grieg Seafood BC Ltd.	Production Director
Graeme Cooper	Grieg Seafood BC Ltd.	Fish Health Technician
Mike Ness	Grieg Seafood BC Ltd.	Fish Health Manager
Steve Munro	Grieg Seafood BC Ltd.	Farm Site Manager
Stewart Hawthorn	Grieg Seafood BC Ltd.	Regional Director
Tim Hewison	Grieg Seafood BC Ltd.	Fish Health Coordinator
Trevor Fraser	Grieg Seafood BC Ltd.	Farm Technician
Cilka La Trace	Marine Harvest Canada	Fish Health Dept
Nils Stein	Pharmaq AS	Accounts North America
Debbie Collins	Centre for Aquatic Health Sciences	
Kim Bull	Centre for Aquatic Health Sciences	
Robert Johns	Centre for Aquatic Health Sciences	
Wyth Marshall	Centre for Aquatic Health Sciences	
Eirik Wilkinson	Pharmaq AS	
Tina Nordstrand	Syndel Laboratories Ltd.	
Tamara Russell	HAMP	
Jeanine Fence	HAMP	

Biography of Presenters

Dr. Michael L. Kent

Since 1999, Dr. Kent has been a Professor at Oregon State University. Dr. Michael Kent was employed at Fisheries Oceans Canada, Fish Health Section, Pacific Biological Station (PBS) from 1988-1999, and was head of the Fish Health Section his last 2 years there. He did a post-doc at the Battelle Marine Lab in Sequim, Washington, from 1986-1988, and this is where he discovered and named "netpen liver disease" from a fish farm in Port Townsend Bay. At PBS, his research was largely focused on diseases of pen-reared salmon, and he has published over 250 papers on fish diseases.

Dr. Barry Milligan, MSc., DVM

Barry Milligan has been a veterinarian and production planner in salmon aquaculture for more than 12 years. Prior to working for Grieg Barry was a small animal veterinarian on Vancouver Island for 1 year, a research associate in Population Medicine at the University of Guelph for 3 years, and a research associate at the Centre for Food Animal Research in Ottawa for 4 years. Barry holds a BSc in Biology from the University of Regina, a MSc in Biology (Marine Ecology) from the University of Victoria, and a DVM from the University of Guelph

Dr. Heindrich Snyman, BVSc., DVSc., Diplomate ACVP

Dr. Hein Snyman is a board certified Veterinary Diagnostic Pathologist specializing in diagnostic Fish Pathology. Hein was born in South Africa and completed his BVSc degree from the University of Pretoria in 2008. After spending some time in private practice in South Africa as an exotics and wildlife veterinarian, he joined the Department of Pathobiology at the Ontario Veterinary College in Guelph as a graduate student. He completed his DVSc in Veterinary Anatomic Pathology in November 2013 and also successfully completed the certification examination of the American College of Veterinary Pathologists (ACVP) in anatomic pathology, September 2013. In December 2013, Hein joined the Animal Health Centre, the BC Ministry of Agriculture's AAVLD accredited veterinary diagnostic laboratory, as veterinary diagnostic pathologist. During this time he developed a keen interest in aquaculture and fish pathology and in July 2014 Hein became the second full time diagnostic fish pathologist with the Animal Health Centre.

Dr. Stephanie A. Smith, Ph.D

Dr. Smith is the co-founder and Chief Scientific and Operations Officer of Beagle Bioproducts, Inc. in Columbus, OH. Beagle was founded for the purpose of providing products and services to people who study, mitigate, and otherwise encounter harmful algal blooms (HABs) and their associated toxins. Dr. Smith developed Beagle's technical capabilities, which include the production of analytical-grade toxins and Beagle's primary service offering, cyanotoxin testing for water and unusual sample matrices. Beagle has been built on Dr. Smith's training in photosynthetic microbiology at The Ohio State Universtiy, where she earned her doctoral degree in 2002. She has served as a Senior Scientist and Associate Manager at Battelle Memorial Institute, as an officer in professional organizations such as the Ohio Branch of the American Society for Microbiology, and as an expert on a panel testifying before Congress regarding the Harmful Algal Bloom and Hypoxia Research and Control Act (HABHRCA). Dr. Smith is an active blogger about HABs, with the aim of raising public awareness and support for the study of HABs, so that technologies and strategies for mitigation can ultimately be developed

Ms Nicky Haigh

Nicky Haigh is a specialist in harmful marine algae, particularly the species that are harmful to finfish. The CEO of Microthalassia Consultants Inc., she is best known as the Manager and Senior Phytoplankton Analyst of the Harmful Algae Monitoring Program (HAMP) in Nanaimo, British Columbia. Building on her education with Dr. Max Taylor at UBC and four years of experience on a BC salmon farm, she developed HAMP, originally with Dr. Ian Whyte of Fisheries and Oceans Canada, to help the BC finfish aquaculture industry with issues of harmful algae monitoring, management and mitigation. In the past 16 years Nicky and HAMP have: helped BC salmon farmers to develop a long-term database of phytoplankton species abundance and diversity; identified new fish-killing species of phytoplankton in BC; and, through annual workshops and online courses on phytoplankton identification, increased the competence of fish-farming personnel on monitoring and identification of harmful algae species. Nicky also works with BC shellfish growers, academia, and government agencies on identification and monitoring of marine algae. She is the author of the *HAMP Harmful Plankton Handbook* (updated annually), and *the Plankton Identification Handbook for Shellfish Growers on the West Coast of Canada*. She has presented research papers at conferences in Canada and abroad.

Dr. Asit Mazumder, Ph.D

Asit Mazumder is a Professor of Biology at the University of Victoria. He is best known for his research in quantifying and modeling how nutrients and foodwebs interact in determining responses of freshwater and marine ecosystems in terms of water quality, nutrient dynamics, foodweb structure, contaminant transport, plant biodiversity and salmon productivity. In partnership with water utilities, communities, academics, government and industries, his research focuses on the integration of inter-disciplinary ecological, engineering, epidemiological and public health sciences into sustainable clean and healthy water for communities. He has published over 125 peer-reviewed papers in international journals including some of the top journals like Science, Ecology, Limnology and Oceanography, Canadian Journal of Fisheries and Aquatic Sciences, Environmental Science and Technology and Environmental Health Perspectives. He had been awarded the Chandler-Misener Award by the International Association for Great Lakes Research for the best scientific paper on the Great Lakes, and the Miller Institute Professorship for Basic Science at the University of California Berkeley. In 2013, the American Society of Limnology and Oceanography (ASLO) awarded him Ruth Patrick Award for his outstanding contributions to aquatic sciences towards environmental solutions for water quality. During the last decade, has been invited to serve as members of several national grant selection committees, as members of Research Management Committees for Research Networks on water, expert advisor to BC Auditor General on water issues and as a member of the panel advising BC Government on Aquaculture Waste Management.

Summary of Presentations

Presenter: Dr. Michael Kent (Oregon State University, Department of Microbiology and Biomedical Sciences)

Title: **Netpen Liver Disease: Pathology, source of the disease & links to Microcystin.**

Dr. Kent's presentation covered the history of the Netpen Liver Disease (NPLD) and its pathology.

In 1986 a sea site stocked with summer time smolts located in Port Townsend Bay was the first documented incident of NPLD. From there the disease was found around Vancouver Island (slide #4 Appendix 1). All fish exhibited significant liver damage. The histopathology of typical NPLD livers revealed severe variability in liver cell size, regenerating liver cells, enlarged cells, severe inflammation around the cells and the presence of hepatic meglocytosis (defining artifact for the disease). Putative causes of the observed liver damage were thought to be caused by PGAs or microcystins. An experimental population of Atlantic salmon under went a Pulp Mill bioassay where they were exposed to effluent from the Battelle, Sequim facility. The disease did not manifest. Progressive investigations from then to 1988 revealed a consistent pattern. The appearance and severity of the disease progresses through the summer and then the fish recover in the winter time except for the persistence of the megalocytes. To this point the disease was identified in Atlantic salmon, Chinook, and Rainbow trout. Transmission studies revealed the disease to be non-infectious. The fish will recover from the disease if moved to fresh water.

Around this time, a UBC lab headed by Raymond Andersen discovered in very high levels the presence of mycrocystin, a known potent liver toxin, in BC mussels. Subsequent testing of livers from fish diagnosed with NPLD at the UBC lab resulted in the detection of a microcystin toxin. This was based on the protein phosphatase inhibition assay using liquid chromatography. Microcystin is associated with the Microcystis cyanonbacteria which is a blue green algae found in freshwater systems. Microcystin was subsequently detected in net biota but the actual source was not determined. Other known algal sources of microcystin have been *Microcystis*, *Anabaena*, *Planktothrix* and, more rarely, *Anabaenopsis*, *Hapalosiphon* and *Nostoc. Synechococcus* and *Synechocystis* from the marine environment. *Oscillatoria sp.* from corals with MC genes.

With the thought that the Atlantic smolts were exposed to the toxin through ingestion of the net biota, an attempt was made to control the condition. A study revolving around sea water entry and feeding rates was initiated at the Pacific Biological Research Station. Feed rates were thought to influence whether the fish would eat copepods. The prevalence of NPLD, mild hepatocellular changes and no evidence of lesions were assessed for each of the treatments. The study revealed a higher prevalence of NPLD in the treatment that had a low feed rate.

In an attempt to reproduce the disease, microcystin was injected into smolt size Atlantic salmon at a concentration of 0.8 mg/kg. The effect was the typical disorganization of the tissue cells in the liver with the obvious presence of hepatic meglocytosis. From this then was the cause of the disease due to microcystin exposure? Was the delivery of the toxic then from fish feeding on zooplankton and net biota or was the source a marine cyanobacterium? With the toxin found in bivalves and the die off of sea otters due to liver toxin exposure, was the source from freshwater runoff? NPLD was also observed in wild salmon. The prevalence of hepatic meglocytosis in BC Chinook and Sockeye was gauged. Spawning Chinook revealed 16% and ocean caught Chinook revealed 7% whereas no evidence of lesions were observed in Sockeye salmon. Another study by Saskida et al in 2007 & 2008 revealed 32-47% in Pink salmon. A survey done in Willamette River, Oregon between 2010-2014 showed approximately 1% of the returning salmon having the defining lesions. Using an ELISA test on samples from pen reared Atlantic salmon and wild Chinook with NPLD, microcystin was detected at a concentration of 0.4 mg/kg and 0.3 mg/kg respectively.

Base on the theory that the source is most likely marine biota, avoidance of the disease would involve ensuring the fish are onto feed shortly after introduction to the net pens as well to introduce fish when zooplankton is not present.

In summary: the disease was seen as a summer event particularly in the first summer upon introduction. The disease is non-infectious. It is linked to feeding on natural biota. It is linked to the toxin microcystin. The fish seemed to recover in the fall. The source was never determined. Chronic effects of the toxin with respect to growth and overall animal health was never assessed.

Presenter: Dr. Barry Milligan (Grieg Seafood BC Ltd)

Title: **Site case study of Netpen Liver Disease in preharvest fish.**

Dr. Milligan's presentation provided a synopsis of an incidence of NPLD diagnosed in a saltwater population of adult Atlantic salmon.

The event was first documented on June 25, 2014 at a farm (designated site A) located in the Nootka Sound region of Vancouver Island. At that time site A contained ~ 3.6 kg fish. The fish exhibited a noticeable drop in feed rate along with a rise in mortality that was fluctuating dramatically with the rise and fall of oxygen levels in the water. There was no evidence of a harmful algal bloom (HAB) as determined by daily sampling at the site along with samples submitted to the Harmful Algae Monitoring Program (HAMP). Histology samples submitted to two separate laboratories resulted however in lesions consistent with NPLD. On site observation of livers from moribund fish ranged from healthly (easily fractured) to shrunken yellow not easily fractured (texture equivalent to beef jerky). Over time, after a 2-3 month period, appetite for the fish improved. Continued monitoring of the livers over time from harvested fish revealed there was a decrease in adversely affected livers and an increase in healthy livers.

Historically, at the company, NPLD was limited to poor – doers at 3-4 months post entry which were presumed not to be ingesting pelleted feed. This new presentation was very different as it was growout fish affected that were established onto feed. Sites in the same area containing smolts (~ 500 grams) did not appear to be affected as the feed rate and mortality remained normal for those populations at the time of the event. Increase monitoring of HAB still revealed nothing abnormal. Histological surveys of neighbouring sites with smaller fish along with fish in an adjacent inlet did not test positive for NPLD.

Based on historical information that the disease has been associated with an heptotoxin mycrocystin-LR (MC), which in turn is known to be produced by an cyanobacteria, efforts where then made to determine the "smoking gun". Beagle Bioproducts is a company that provides products and services specialized in harmful algae. Tests employed to screen for the presence of mycrocystin were ELISA (developed specifically for water samples) along with HPLC and Mass Spectrometry for confirmatory means. Water samples from the affected site along with samples taken from the Sechelt area, the opposite side of Vancouver Island, were submitted for testing. All samples tested positive for the presence of MC with the surprising result of the Sechelt samples, thought to be a negative control, having higher concentrations.

Presenter: Dr. Heindrich Snyman (BC Ministry of Agriculture, Animal Health Centre)

Title: **Histopathology of chronic liver disease in Atlantic Salmon raised in Nootka Sound in 2014.**

Dr. Snyman's presentation covered the histopathology testing completed on a population of farmed Atlantic salmon observed to have clinical signs of NPLD.

The liver in fish is ventrally located in the cranial coelomic cavity. The largest organ in the fish, it is not a lobed organ (unlike humans) but does contain the characteristic internal sinusoidal structures encompassed by a double cell layer of hepatocytes. Blood enters the liver through the hepatic artery and portal vein and flows out through the hepatic vein. The classic hepatic lobule is not apparent in fish but the functional units (hepatocytes, hepatic sinusoid, bile ducts, central vein, heaptic arteries and veins) are still present. In fish, the liver still takes on the role of nutrient metabolism, digestion (via bile), protein synthesis as well as the detoxification and excretion of fat soluble toxins. The biotransformation of the toxins occurs in 2 phases: 1) the use of cytochrome P450 enzymes for oxidation, reduction, hydrolysis, hydration, and dehalogenation 2) conjugation pathways for sulfation, glucoronidation, glutathione conjugation, methylation, acetylation and amino acid conjugation. The resulting waste material is then eliminated through the gall bladder and kidneys.

A sample submission on July 2nd 2014 of multiple organs and tissue from 5 recently dead fish was received at the Animal Health Centre, Abbotsford, B.C for histopathology testing. The samples originated from a population of preharvest size Atlantic salmon located in Nootka Sound on the west coast of Vancouver Island (designated Site A). Four out of the 5 fish had characteristic histological lesions of NPLD with the remaining sample having signs of early hepatotoxicity consistent with early and potentially reversible NPLD. A follow up sample on July 7th from another site in the same region with a similar clinical presentation (Site B) resulted in 5/5 fish with NPLD lesions. The clinical presentation of both cases did not fit the classic description as these samples were from pre-harvest fish; there was a greater population effect, and no suppressed feeding as the fish were well on pelleted food. To assess population effects a multiple organ sample set from 5 fish from the living population at site B was submitted for testing on July 8. One of the 5 fish had lesions severe enough to justify a diagnosis of early NPLD with the remaining 4 exhibiting similar (but milder) evidence of low-grade hepatotoxicity.

Common lesions associated with NPLD in these fish are: Increased Pigmented Macrophage Aggregates (PMP), Hepatocellular Megalocystosis and Karyomegaly (MEG), Biliary Preductular Cell Hyperplasia (BPH), Perivascular and Pericholangiolar Inflammation (PVL & CPL), Hepatocellular Hydropic Degeneration (HHD), and hepatocellular single cell necrosis (SCN). PMP are normal structures found in the liver, kidney and spleen which contain lipofuscin and hemosiderin pigments (cellular breakdown products). The accumulations of these pigments are common indicators of chemical and non-chemical stressors. Presence of these pigments can be used as a chronic exposure marker (Appendix 3, slides 15-19). MEG are characterized as grossly enlarged hepatocytes with an enlarged and hyperchromatic nucleus. They provide evidence of sub lethally injured cells and although functionally impaired these affected cells might be able to survive for several months. Several types of toxins including: aflatoxoins, pyrrolizidine alkaloids, complex chemical mixtures from marine sediments extracts and the algal toxin microcystin-LR have been linked to the genesis of these cells. Presence of these cells can be used as a chronic exposure marker (Appendix 3, slide 21-22). BPH occurs during cellular regeneration. It is evidence of exposure to toxins and can be used as a chronic exposure marker (Appendix 3, slide 23). PVL and CPL is the inflammation observed around the blood vessels and bile ducts within the liver. It is considered a non-specific immune response to antigenic stimulation. This type of inflammation can be used as a chronic exposure marker (Appendix 3, slide 25-28). HHD is evident when cytoplasm of affected hepatocytes is expanded by fine to large foamy vacuoles. Sometimes the vacuoles coalesce and are larger than normal hepatocytes. HHD is evidence of reversible acute cellular damage (occurred within 6-12 hours). Differentials include exposure to toxins (endogenous or exogenous). Single cell necrosis (SCN) occurs when it is no longer reversible. This can be an acute exposure marker of an ongoing injury. (Appendix 3, slide 29).

With the observation of all these lesions in the samples submitted in July, the diagnosis was then given for NPLD. The samples showed both evidence of chronic disease and acute ongoing injury.

To asses population prevalence additional samples were taken after the initial event from site B from harvested fish at the processing plant on July 16, 28, and August 20. Twenty livers from each date were scored for lesions (none (0), mild (1), moderate (2), severe (3)) then averaged. As expected chronic marker lesions remained present with a general increasing trend as time progressed. Acute and ongoing injury markers (HHD) were consistently present as time progressed as well (Appendix 3, slides 36-38).

Additional samples were also taken after the initial event from site A from harvested fish at the processing plant on August 8 and August 20. Again Twenty livers from each date were scored for lesions (none (0), mild (1), moderate (2), severe (3)) then averaged. Chronic marker lesions remained elevated as time progressed along with the persistence of acute markers (Appendix 3, slides 40-42).

History repeated itself in November 2014. At two additional sites (C & D) NPLD was detected in 3/5 and 4/5 respectively. A prevalence assessment was completed at site C where 6 pens, 20 fish per pen were sampled for histopathology testing. Variation in severity of lesions was seen between pens especially with respect to SCN being present in 3 pens which also had higher scores for PMP, PVL, and HHD (Appendix 3, slides 45-47). For site D, 4 pens, 20 fish per pen were sampled. Again, a pen variation was observed and lower scores occurred compared to site C (Appendix 3, slides 49-51). This observed pen variation might suggest variation in exposure even across a small space such as a single netpen.

Chronic liver disease (NPLD) was present at all 4 sites. There was evidence that there was an increase in severity over time along with evidence of ongoing hepatocellular injury. The increase in severity could be attributed to the failure of the liver to excrete endogenous circulation toxins due to aberrant liver function. Alternatively the hepatocellular injury could also be attributed to an ongoing low grade exposure to an environmental hepato-toxin. The type of toxin; whether it is microcystin or one of its congeners, is in question along with the route of exposure (oral being most likely but gills, cutaneous might also be considerations).

Presenter: Dr. Stephanie Smith (Beagle Bioproducts Inc)

Title: **Looking for Microcystin associated with Netpen Liver Disease: Does ADDA add up.**

Dr. Smith's presentation focused on the microcystin toxin and the testing used for the detection of the toxin.

The microcystin toxin is a freshwater cyanobacterial toxin associated with cyanobacteria genera such as *Microcystis*, *Plantothrix*, *Anabaena*, *Aphanizomenon*, and *Anabaenopsis*. There is at least one marine example, *Synechococcus*. It is a liver toxin that inhibits protein phosphatases and is widely thought to be carcinogenic. There is data that shows the possibility of chronic poisonings in humans (not proven) and acute poisoning in humans are very rare, mainly limited to cases at a dialysis centery in Brazil in the 1990s. Acute poisoning have occurred in other animals especially birds and dogs. There are over 100 congeners (example: LR, RR, WR). In literature Microcystin-LR is the most commonly studied; however, Microcystin-RR might be more prevalent based upon what Beagle has found in the American Midwest. ADDA is a β amino acid within the structure of all congeners of microcystin and without it the toxin is not potent. Alone ADDA is not toxic (that we think). ADDA is also present in Nodularin. Conveniently ADDA absorbs at 238 nm so this structure is our best tool in tracking toxins in water samples, using tools that measure absorbance. There are also antibodies specifically to the ADDA moiety which are used in ELISA tests.

For analysis of microcystin it is best to consider both the cost and accuracy of the test. The test routinely used is HPLC-PDA (high performance liquid chromatography with photodiode array detector). The gold standard test is considered to be LC-MS (liquid chromatography-mass spectrometry) for detection and identification. This test requires very expensive equipment and highly trained staff, and sample preparation is extensive. PPIA (protein phosphatase inhibition assay)is a highly sensitive test that can be used for screening but lacks specificity compared to some other tests, because the enzymes might be inhibited by other things in the samples. The ADDA ELISA test is easier and more affordable (relative to the other techniques) and can be used as a screening tool. For the 96 well test plate format the cost is $450/plate and requires a plate reader or spectrophotometer. This test is now required for water testing in the state of Ohio due to the 2014 toxin event in Toledo. The ADDA ELISA is an indirect competitive ELISA. The more microcystin present in the sample, the less primary antibody will bind to the coated plate. The polyclonal antibody used in the test was manufactured against BSA-linked ADDA. The test was

developed for water samples hence not so accurate on tissue samples and dirty water. "Matrix effects" with such samples make them prone to false positive or false negative results: positive with the anitobodies erroneously bind to non-ADDA targets, and negative with other things in the sample prevent the antibodies from binding to ADDA at all. Recent commercial tests are now available using monoclonal antibodies against ADDA along with a new direct monoclonal ELISA (Preece et al, 2015); it is presumed that these are less prone to matrix effects.

Beagle Bioproducts launched a microcystin testing service in May 2014. The company was involved with the testing following the massive bloom in August 2014 off the shore of lake Erie which resulted in 2 ppb of microcystin toxin in finished water causing a "do not drink" advisory to 0.5 million people. After launch, the company received numerous requests to extract and test odd products (flour, beer, and fish livers!). First, an extraction method was developed for the fish livers by homogenizing the tissue in methanol. The extraction process was allowed to occur over a 3 day period at 4 ˚C. A hexane liquid-liquid extraction step was added for clean-up in order to achieve the right balance between yield and low background. Samples were then dried and reconstituted using water in a ratio such that a 1 ppb result from the assay would reflect 1 ppb in the tissue sample.

Healthy livers from fish not originating from the affected sites (East side of Vancouver Island) were requested to be used as presumed negative controls for the testing. Some of the healthy liver samples were spiked with microcystin prior to extraction to serve as a positive control for the whole process of extraction and testing, and also to estimate yields of the process. Dilutions were often required so that the amount of microcystin present would be within the range of the standard curve on the ADDA ELISA. Initially, positive results were seen from both the negative and positive controls. Further dilutions saw an increase in values (Appendix 4, slide 13). This phenomenon suggested then the presence of inhibitory substances in the tissue samples (i.e. a matrix effect) preventing the detection of the toxin. As the sample is diluted so are the inhibitory substances revealing the true presence of toxin. However, too much dilution could produce false negative results if the toxins are present at low concentrations. Livers samples from NPLD affected fish were tested and one (diseased liver #5) produced a clear signal at 1:1000 dilution (Appendix 4, slide 14). Muscle (flesh) samples were similarly treated and revealed no toxin to be present (Appendix 4, slide 15). The step involving drying the samples was bypassed and diluted extracts were tested directly (Appendix 4, slide 16). Expected values of 3500 and 1500 ppb from spiked liver and muscle respectively were seen showing good recovery during the extraction process. Again a positive hit was seen from diseased liver #5. Questions arising from these results were: 1) why are the healthy livers showing detectable amounts along with some of the diseased livers? 2) At least one diseased liver (#5) is a clear hit – why not all of them? For the healthy and diseased liver results it could be that the matrix effect is not being completely eliminated by dilution. Or, fish with the healthy livers have been exposed but did not succumb to the disease (lower level of exposure or for some other biological/physiological reason like age). Biodilution could be a factor since only one of the diseased liver producing a measureable amount of toxin.

The PP2A assay is an enzyme-specific assay for the detection of microcystin from which we would expect numbers from this assay to mirror that of the ADDA ELISA. The ADDA ELISA suggests the diseased livers tested with the exception of one had more microcystin than healthy livers (Appendix 4, slide 18). The PP2A assayed confirmed the diseased liver #5 had more microcystin than the healthy livers as well as the other diseased livers. Extracts of the healthy livers when ran on HPLC-PDA again showed a high degree of matrix effect, but a peak in the area where we might expect to see Microcystin-RR led to further analysis. Healthy liver samples were spiked with microcystin-RR (MC-RR), and along with a MC-RR control, the diseased liver referenced above and a healthy liver not spiked were ran the HPLC-PDA again, with measurement at 238 nm. A significant peak was seen with the healthy liver, diseased liver, and MC-RR control (Appendix 4, slide 20). However when fractions from the corresponding peaks for these samples were tested using mass spectrophotometry (MS), the ions did not correspond to that of MC-RR. This could indicate the presence of another congener or it is not microcystin-RR but rather is a microcystin of similar hydrophobicity which therefore migrates with MC-RR. Another approach that is in development is to indirectly measure total microcystin by chemically cleaving the ADDA moieties from the toxin to yield MMPB (2-methyl-3-methoxy-4-phenylbutanoic acid), followed by liquid chromatography and MS. Using

this approach, for diseased liver #5 we saw detectable amounts of MMPB confirming both the ELISA and PP2A results which showed the presence of microcystin.

Attempts were made to determine the possible source of the toxin. Water samples taken from the affected sites, taken from a fresh water source in the area of the event, a feed sample from the inventory fed at site B (cross reference with Dr. Barry Milligan and Dr. Heindrich Snyman presentations), and net scrapings from site A were all tested by the ADDA ELISA. Water samples from two of the affected sites (A and B) and from Vancouver Island West produced signals at the low limit of detection or barely above, and were not convincing evidence of the presence of microcystin. Conversely, a second set of water samples, net scrapings and caprellids from the East side of Vancouver Island resulted in detectable amounts of microcystin.

In summary, the ADDA ELISA was used to detect microcystin in livers from the farm sites; however, the toxin appears to be present in both healthy and diseased livers. The ELISA test should be viewed as a screening tool with these tyes of samples. The matrix effects from the tissue samples do muddle the results especially to detect lower concentrations of microcystin. The PP2A assay and detection of MMPB supported the presence of microcystin in diseased liver 5. Most water samples had no microcystin or microcystin near the detection limit of the ELISA assay. Net scrapings and caprellid samples from the east side of Vancouver Island had detectable amounts of microcystin. Better methods to detect the toxin in tissues need to be developed. Testing of new kits and further development of the HPLC test for tissues would be recommended for future samples.

Presenter: Ms Nicky Haigh (Harmful Algae Monitoring Program)

Title: **Toxic Cyanobacteria & their possible role in Netpen Liver Disease.**

Nicky Haigh's presentation focused on the algae found at the saltwater sites and their relation to NPLD.

Cyanobacteria are also known as blue-green algae. Despite being call algae, the organism is a prokaryote deriving its energy through photosynthesis. They are either single cells, filamentous or found in colonies. There are both planktonic types and types which will grow on surfaces such as nets or other algae. Species of cyanobacteria can be found in the marine, brackish, and freshwater environments.

Toxic cyanobacteria are mostly found in the freshwater environment. They can produce a host of toxins including neurotoxins as well as hepatotoxins such as microcystin. *Microcystis* species are found in both freshwater and brackish water types and produce microcystin toxins. *Lyngbya* species are found in the freshwater and marine environments. This genus is associated with neurotoxins and other toxins. *Oscillatoria* species inhabit freshwater and marine habitats. There is some question as to whether or not these species manufacture microcystins or other toxins. *Nostoc* species have been found in the freshwater, marine and terrestrial niches. Microcystin and neurotoxins have been linked to this genus.

The possible roles that cyanobacteria could play in association with NPLD are direct consumption, secondary accumulation, or run-off exposure from freshwater blooms. *Nostoc* species do form larger colonies that may inhabit the surface of the nets. Fish could then directly graze off of the nets fouled with these colonies. Some *Nostoc* colonies are brown in colour and may become free floating thereby resembling feed. There are amphipods, e.g. *Caprella* species that graze on algae. These crustaceans could then accumulate any toxins found in the algae which in turn could then be ingested by the salmon. Thick blooms of *Microcystis* have been known to occur in streams. There is the possibility then for high water flow with spring rains scouring the streams bringing the algae into the marine environment. This in turn could have an affect nearby farmers.

Recommended next steps would be then: 1) Sampling of the net algae for the presence of any species that might be associated with microcystin. 2) Sampling of Caprella for the purpose of monitoring for

presence and levels of microcystin. 3) Water samples to be taken at the affect sites and sites not affected by the disease for toxin analysis. 4) Microscope analysis of water and plankton tows taken at affected sites and sites not affected by the disease.

Presenter: Dr. Asit Mazumder (University of Victoria, Department of Biology)

Title: **Causes & consequences of algal toxins in aquatic systems and food webs.**

Dr. Mazumder's presentation outlines the impacts and ramifications of increase nutrient loads into aquatic systems

Anthropogenic inputs of phosphorus and nitrogen into aquatic systems lead to eutrophication and associated algal blooms. Ramifications result in loss of aquatic diversity and loss of habitats due to anoxia conditions. With the presence of toxic algae, fish populations are threatened along with impacts to human health. Large algal blooms also provide a substrate for waterborne pathogens. Increase counts of these bacteria means an increase in chlorination by products amounts in drinking water that are harmful to humans.

These algae blooms are made up of cyanobacteria (blue green algae), dinoflagellates and some diatoms. Products of concern from these blooms are: neurotoxins (alkaloids, non-protein amino acids), hepatotoxins (peptides), and lipo-polysaccharides. Algae attributed to these toxins include Nostoc, Anabaena, Oscillatoria, Aphanizomenon, and Microcystis. Symptoms cause by these toxins include neuro-degeneration, vomiting, respiratory blockage, and diarrhea. Recent medical results have shown some of the neurotoxins can even bio accumulate in tissues and the nervous system.

The fast growing global population leading to the increase in demand for food has been linked to: 1) sever water shortages and 2) increase in nutrient loading to fresh and coastal marine water. For example; one apple requires 70 litres of water, 1 kg of barley requires 1300 litres, and 1 kg of beef requires 15500 litres. The increase use of nitrogen fertilizers has kept pace with the demand for food as seen in global trends (Appendix 6, slide 6-7). The increase presence of phosphorus in water systems is directly linked to the increase in chlorophyll (Appendix 6, slide 8). While these two chemicals play apart in the proliferation of algae, it is not only the amounts of these nutrients but the composition of these nutrients with respect to the ratio present that determines the types of algae that form blooms (Appendix 6, slide 12-13). Factors influencing this ratio include seasonality of nutrient inputs, physical properties of the receiving systems and the structure of the foodweb in the receiving systems.

There is global evidence of neurodegenerative and hepatodegenerative disease caused by toxins in cyanobacteria. This is seen among the Chamorro people of Guam, linked to ALS among Gulf War veterans and linked to ALS patients in Annapolis Maryland USA. There are also recorded events of animals killed by microcystin toxin exposure from algal blooms like the otters in Monteray Bay. The contamination was sourced from freshwater run off from Pinto Lake where the bloom occurred. Further evidence can be seen in the increase of shellfish poisoning (PSP, NSP, DSP, and ASP) along coastal ecosystems due to increase in algal blooms as a result of increased nutrient addition from agriculture activities being transported to these ecosystems. With the increase in algal toxins in the saltwater, it begs the question can these toxins bioaccumulate in fillets that people would consume?

Summary of Discussions

Questions from: **Netpen Liver Disease: Pathology, source of the disease & links to Microcystin**

What about NPLD and sockeye salmon?

Not seen in sockeye. Some fish are resistant for example Tilapia.

Detected and reported in Washington State and BC. Any other parts of the world have seen something like this?

To 1999, not seen elsewhere- only Pacific NW.

Any evidence of neurotoxic substance?

Not that I know of.

Microcystin was found in mussels. Are the mussels producing it or just concentrated?

Concentrated. A good source to look for the toxin in the area affected.

Did you detect the cyanobacteria?

No, did not find any up to the point where the research stopped and I left BC. That was the end of funding for the project. Now people are looking for mycrocystin genes which would probably be a more effective thing to do.

Do you think that mycrocystin is the cause or is that the one that is known?

Good point. Do need to keep an open mind. It could be linked or perhaps there is some other toxin. Yet the disease can be induced by injecting fish with mycrocystin. Never did any feeding studies. Crab larvae with microcystin were fed to fish but the liver disease is not as profound. A lot of questions.

Would it be hard to screen for a wide range of causes (toxins)?

C*hemist could provide the different assays. Bioassay could be developed.*

Has anyone tried any similar injection studies with cylindrospermopsin or nodularin?

No, not that I am aware of.

What kind of timeline from injection to lesion?

Acute form of necrosis (overdosing the fish) within days. Else a week to 10 days (0.8 mg/kg dose).

How do you define the megalocytosis?

They are not dividing. They are in arrested development. The hallmark is the nucleus is dramatically larger than the surrounding cells. Two categories: Nuclei are 10x the diameter of the normal cell. Cells appear dramatically bigger. (AHC definition: Nulcei are at least two to three times normal)

Could you describe more the lesions you saw in adult Chinook?

The fish were returning to fresh water and not feeding. Most of the livers from the fish looked normal but approximately 1/100 would have lesions that appear as chronic not acute.

Could you determine when they (adult Chinook) were exposed?

Difficult to say. The lesions are always chronic.

Any juvenile salmon in the area that had the disease as well?

No, did not look at the juveniles. Good point to look into though. We looked at a fair amount of shiner perch. Never saw the disease in the shiner perch.

How long does the toxin persist in the environment?

I don't know.

Questions from: **Site case study of Netpen Liver Disease in preharvest fish.**

Was there sampling of juvenile salmon in the area for signs of the disease?

Yes, there has been sampling over the past 3 years for sea lice data. Tissue samples have been banked from the last 2 years but we have not quite decided what to test for. Good suggestion to see if the fish in the surrounding environment also experiencing the disease. We could see if the issue is localized to the site or is widespread. Could then tell you the possible source of the toxin (local or from elsewhere). Everywhere we looked for the toxin it was found except for the hatchery. It was a surprise.

Do we know of the levels of microcystin that would be toxic?

Will leave that for Stephanie to discuss. The ELISA used is designed for a water-based test not for tissue assay.

Questions from: **Histopathology of chronic liver disease in Atlantic Salmon raised in Nootka Sound in 2014.**

The markers that you used, one feature leads to the next, what was the most reliable in your opinion?

The inflammation is really not specific. The most significant lesion would be the acute degeneration of cells. The best indication that there is ongoing and recent exposure. Towards the end they (fish) were starting to do better even though the lesions were starting to increase There may be some other factors.

-continued- Exposure throughout the whole period?

Yes, it could be. It looks like there is something going on throughout the whole period. And then it carried on, getting a little bit worse. Would like to test if taken to fresh water would there be to full recovery.

Open Question period for the first 3 presentations:

Does this not fit with ingestion of sea water since being chronically exposed to something? Fish in sea water are constantly drinking sea water thereby filtering that (the water) out and being absorbed directly to the liver. The prime target is there. Are we talking about the ingestion of water?

Hein Snyman: *I would agree. We don't know.*

Mike Kent: *Only one experiment was done with fish put into sea water tanks on the docks. The fish were exposed to the same sea water as the cage fish. It was a controlled experiment which would bear repeating*.

What was the water column doing? How was it distributed? Are there certain streams in these areas? Where will microcystin be found?

Nicky Haigh: *Do not feel it is something that is ingested through the water (chronic exposure).*

Why not all of the above (toxins and algae in the water). We should not exclude. There has been mining and logging activity in the area. Perhaps the net pens are the source of it (accumulated toxins).

Is it related to farming practices or is it related to something else?

Stephanie Smith: *Personally I have sampled many blooms of microcystis that are not toxic but the genes are there. The best evidence I've seen is that the toxin plays some sort of protective role against reactive oxygen species. There is some evidence that the toxin covalently binds to denature proteins and it becomes part of the pathway to get rid of crappy proteins within the cell. I think there is some evidence emerging that there is a protective effect. Stressed cells are more likely to make the toxin. But you will find stressed cells that are not making it. Is a very tough question that is wide open.*

Stewart Johnson: *Can grow these cultures (algae) and they are not toxic. But the genes are there. Where they were in the growth cycle, could make them really toxic*. *There are certainly questions about toxin and algae. Stressor. Variant in salinity? Extremes of salinity? Toxins could be apart of the common flora that is on the nets*.

Why one inlet and not the others?

Mike Kent: *Nutrient stress? Different geographic conditions?*

Are the toxins being produced in the net pens or transported into the net pens?

Stephanie Smith: *I personally feel that there is something going on with transport. If we look at what happened at Monterey Bay with the sea otters. It is being transported from the river into the bay, concentrated in the mussels, the otters are eating the mussels. That is transport issue. You can test that on site with sampling mussels.*

Barry Milligan: *No signs of cyanobacteria in our water samples or net scrapings sent to HAMP. We do see NPLD once in a while. Usually not a huge issue. It is very unusual something must have changed from before. The one thing we noticed is that the water had a different look to it, almost glacial-like run off. First time we saw that the freshwater lens about 2 metres deep. Seeing that type of water again this year. Feeding verses drinking – how do you get 800,000 fish to eat enough by infusion? Doesn't seem likely? The fish were stressed and at the surface finning not interested in feeding. There was a subpopulation all the way through harvest like that. Whenever there was a drop in salinity there would be a spike in mortality. This could be caused by the chronic affect of the disease?*

There is nothing normal about the water on the west coast. How do you tease out any contributing factors to the disease?

Marc Trudel: *Last summer the water temperature in the North Pacific Gulf of Alaska was 7 degrees Celsius warmer. In Oregon the water was 2 degrees Celsius warmer. The trend of warmer water in the Gulf of Alaska is continuing this year. Very abnormal situations. The Tula foundation Calvert Island have seen 4 degrees surface temperature higher than last year.*

Kathleen Frisch: *Cermaq had an issue (NPLD) in the winter of 2013 following a low DO period. Not sure if it was a bloom. It was a different situation in terms of location, water temperature, salinity, and plankton yet a very similar case*.

Did you have NPLD diagnosed in fish?

Kathleen Frisch: *Yes. In December 2013 to January 2014 (Cermaq)*.

Questions from: **Looking for Microcystin associated with Netpen Liver Disease: Does ADDA add up.**

The use of monoclonal antibodies in kits usually lowers the sensitivity of the assay while increasing the specificity. Do you think this would be an issue for testing these types of samples?

I think there is that risk. The lower range in the samples is 0.4 so a little less sensitivity in the assay will not have that much affect.

Have you also tried any blocking agents?

No, we don't have the freedom to do that. The kit has the blocking agent and the buffers so any addition would be outside of the quality control of the kit.

Question for DFO. Has anyone started testing the net pens in Georgia Strait?

Unknown

Intrigued with the chemistry involved with cyanobacteria. Can it (the toxin) be found in something that is not cyanobacteria?

I think that it is highly improbable but not impossible. Four – 6 genes involved in these bacteria. I think some of these may be bioderivitized.

What we see in cytotoxin is that it transforms as it goes up the food chain becoming more toxic.

Continuing to use ADDA as the primary target makes sense as that piece of the molecule not will not change. I believe too that transport is playing an important role in how it gets to these sites.

Is the ADDA antibody available for purchase?

Yes, you can get it from a Chinese company (patent rip off).

Mike Kent: This could be used then in histochemistry. The antibody then could be labeled and work with Hein to do the histology. This could then be correlated with archived material of both wild fish and farmed fish from the past (1988-1992). The blocks are still at DFO (PBS, Nanaimo). These toxins are stable. Nothing more definitive than finding the antibody in the liver.

Have seen a paper where the microcystin was labeled so would be able to follow where it went in the tissue.

The challenge with that is it takes a lot of toxin. The cost would be in the thousands. Hard to get enough toxin to label.

Questions from: **Toxic Cyanobacteria & their possible role in Netpen Liver Disease.**

Is there Nodularia here on the west coast of B.C.?

Unknown. Haven't seen it to date.

Is there a possible timeline? Is it possible that these things bloom weeks or months before?

It is possible but we do have regular sampling done at the sites. We don't see anything resembling the species talked about at the start of the presentation. There may be marine algae that produces microcystins but seems to be more likely benthic or growing on the substrate.

For the Caprellids, any experience to see what types of algae they are eating? Compare what is inside the guts?

Maybe but have not tried. It is possible to do.

Do you normally check for all these species (routine sampling?)

In the routine water samples we look at all of the blanket species.

Is there a seasonality to the toxin (i.e Spring run-off)?

We do not know though it appears to be persistent.

Mike hinted that marine species produce microcystin. Are there any species that may be possible candidates?

Cyanobacteria species – Oscillatoria and Nostoc

Has any PCR testing been done to ID what kind of cyanobacteria is there?

No. Perhaps stomach lining and the gills would be worth looking at.

Questions and discussion from all presentations:

The idea of feeding fish charcoal was brought forth. Since charcoal has the properties to bind toxins would it be possible to manufacture a feed with 10% charcoal. Along that lines there is also a drug called cholastorimine. It has been used for decades to treat high cholesterol but it is also known to bind microcystin in the stomachs of dogs

With Industrial pollutions from pulp mills, has there been any studies about the longevity and persistence of chemicals and toxins from the mills?

Jim Powell: *BC CAHS Project was to look at Malachite green residues in ground fish as an estimate of 2 tonnes a year coming from mills. Longevity and persistence depends on what chemicals are coming in and depends on the ½ life of the compounds after they entered the environment.*

A question with regards to climate change, with more rain than snow is there a greater chance of algae blooms?

Nicky Haigh: *No, we see blooms every year*

Was stomach content analysis done last year and to be done this year?

Barry Milligan: *No, we should be though and it could be done*

Kathleen Frisch: *When we examine fish that died of this when you open their guts you see feed in them. Need to know what happened 2-3 weeks before that rather than the day before.*

Since the fish (non-affected) eating the pellets do well it is unlikely to be related to the feed. What concentration of water do the fish have to ingest to get the effect (of the toxin) of gulping water?

Michael Kent: *Fish drink a lot of water (have to in salt water) and secret those salts as concentrated urine. Are they effectively a filter? Could be a low concentration in the water if constantly gulping.*

No smoking gun. Is it a toxin? What type of toxin might it be? Have they done TDI work in cows?

Stephanie Smith: *Don't know. There is TDI (total daily intake) for pigs by the World Health Organization. It takes a lot of toxin to do a TDI.*

Heindrich Snyman: *Based on the histopathological signs there are some tests in ecotoxicology studies that are available*.

There was the observation of glacial input at the affected sites?

Barry Milligan: *Yes, last year looked like glacial run off.*

Certain areas can vary greatly. Global warming aspects result in a lot more rock visible every year with the glaciers shrinking. What impact does that have? What about local mining and forestry activity?

Nicky Haigh: *We have seen the nutrient ratio change but would be surprised to see a great change.*

Mark Trudel: *Glacial could increase clay particles in the water. Last year there was a lot less toxic bloom activity on the west coast. The warm blob prevented upwelling on the west coast. There was less surface salinity last year as well. Not the usual influence we see.*

Trevor Fraser: *There has been increased Forestry activity in the area.*

How long has the farm been active in the area where the phenomenon happened?

Barry Milligan: *2003*.

What was unique about the area in 2014?

Barry Milligan: *Last year was warmer. Appear to be more sediment in the water according to visibility measurements.*

Are there any significant things that are the same as these sites (Grieg) for Cermaq?

Kathleen Frisch: *Same low DO rates in the December of the year prior in the Tofino area. No drops in salinity though.*

Barry Milligan: *There were 2 freshwater incursion (run-off) events*.

Fish are seen as a great sink for phosphorous in a lot of FW systems. Is that the case here?

Kathleen Frisch*: Phosphorous levels are very low and not comparable (in saltwater). The saltwater environment is different than freshwater with that respects*. *There is a lot of flushing of the ocean in the farming areas*.

Are there freshwater cyanobacteria blooms in the local watershed?

Stephanie Smith: *Not too sure if anyone is looking at this*.

Summation

Evidence points towards the presence of microcystin as the causative agent but no confirmation has been made. Immunochemistry would provide this confirmation by using an antibody derived against ADDA, rather than specific congeners. Better tests to determine levels within tissues need to be developed in order to get around the matrix affect.

The idea of ingestion of water as part of the fish's process of osmoregulation has merit to be followed through with an experiment

Histology markers indicate the possibility of an on going exposure. Monitoring water samples to establish base line of toxin levels in the area and nearby fresh water run off. As filter feeders, mussels may bioaccumulate the toxin so it would be valuable to include them in the sampling survey. Sediment may be a valuable parameter to include in the surveillance.

For mitigation, if the ingestion theory proves to be, then the possibility of additives to the feed (charcoal or cholastorimine) should not be forgotten.

Research and Development Priorities

<u>Research Project 1</u>

Objective

To determine the presence or absence of the ADDA molecule through immunochemistry in affect liver tissues. The development of a fluorescene labelled ADDA antibody can then be used in a test on liver tissues with lesions associated with NPLD. Results from this testing would confirm lesions associated with NPLD are cause by a microcystin toxin. This test could then be applied to future samples from both farmed and wild-stocks suspected of microcystin exposure.

Outline

Generate positive control liver tissues through exposure to toxic microcystin (ADDA present) as well as adequate negative control samples.

Produce a stock of fluorescene labelled ADDA antibody and adapt its intended ELISA use for immunohistochemistry (IHC).

Optimize IHC protocol to determine 1) working dilution of the labeled antibody 2) optimal preservation method of tissues (Davidson's vs Formalin).

Test histology samples archived from recent NPLD events and from described 1989-92 events to establish a causal relationship.

Research Project 2

Objective

To determine a possible means of delivery of the toxin into the fish. The "gulping" experiment will investigate the theory that the toxin is ingested by the fish. Results from this test would then confirm the presence of the toxin in the water column inhabited by the fish. This information can then be applied to mitigation strategies and water surveillance programs.

Outline

- Microcystin at a concentration of xx/L
- 5 experimental tanks stocked at the appropriate density of saltwater conditioned fish
- 1 tank control receiving no feed and no toxic exposure
- 2 experimental tanks receiving no feed but exposed to the toxin
- 2 experimental tanks receiving feed and exposed to toxin

The experiment will be conducted over a 14 day period. Histology samples of the livers from each treatment will be taken at the mid point (day 7) and at the end of the experiment using the optimal preservation method determined in Project 1. Livers will be screened for the presence of lesions associated with NPLD.

Project 3

Objective

To develop a water and mollusc surveillance program in order to establish base line levels of toxin in the sea sites areas affected and near by freshwater influences.

Outline

Drafting sampling protocols for both water and molluscs which will outline sampling methods, sampling points, and frequency of sampling. The collection of information will be used to generate a database. This bank of data can then be used to establish base lines to expose what would be considered elevated amounts and trends.

Appendix

Appendix 1 : Netpen Liver Disease: Pathology, source of the disease & links to Microcystin.

Appendix 2 : Site case study of Netpen Liver Disease in preharvest fish

Appendix 3 : Histopathology of chronic liver disease in Atlantic Salmon raised in Nootka Sound in 2014

Appendix 4 : Looking for Microcystin associated with Netpen Liver Disease: Does ADDA add up

Appendix 5 : Toxic Cyanobacteria & their possible role in Netpen Liver Disease

Appendix 6 : Causes & consequences of algal toxins in aquatic systems and food webs