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Christine Klykken

Nephrocalcinosis in juvenile farmed Atlantic salmon

Doctoral thesis

NTNU
Norwegian University of Science and Technology
Thesis for the Degree of
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Preface

The following thesis was submitted in fulfilment of the requirements for the Degree of Philosophiae Doctor (PhD) at Faculty of Natural Sciences (FNS) under Norwegian University of Science and Technology (NTNU), Trondheim, Norway. Original research included in the dissertation was completed during a four-year industrial PhD-project (NæringsPhD) funded by the Research Council of Norway (project number 304498) in collaboration with Aqua Kompetanse AS. The project also received funding through Norwegian Seafood Research Fund (FHF) as a part of the project NEFROSMOLT (project number 901587).

The project team consisted of the following members:
Christine Klykken, MSc, FNS, NTNU & Aqua Kompetanse: PhD student
Rolf Erik Olsen, Professor, FNS, NTNU: main supervisor
Kari Attramadal: Førsteamanuensis II, FNS, NTNU: co-supervisor
Lauris Boissonnot: PhD, Aqua Kompetanse: mentor
Torolf Storsul: Cand.med.vet, Aqua Kompetanse: project leader

I would like to thank my supervisors Rolf E. Olsen and Kari Attramadal for their guidance, encouragement and scientific discussions throughout my PhD project. I would also like to thank my excellent team of co-authors involved in my project; Alf Dalum, Anne K. Reed, Morten K. Moe, Paul Whatmore, Essa A. Khan and Camilla Karlsen. A special thanks to my mentor and friend Lauris Boissonnot for helping me bring out the best in my work.

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Last, but not lest, I wish to thank my family for their support and encouragement, and healthy distraction throughout my PhD project.

Christine Klykken
Namsos, June 2023

Summary

Nephrocalcinosis has been reported as the current most important risk factor for production of Atlantic salmon smolts. The condition was first described in Atlantic salmon in 1999 and is characterized by mineral deposits in the kidney tubules and collecting ducts. In humans, nephrocalcinosis is a multifactorial disease, and it is likely that this is also true for farmed fish. Nephrocalcinosis has been reported in a wide range of different fish species, both for FW and SW species, but primarily in aquaculture and not wild populations. It is a common consensus that nephrocalcinosis is linked to intensive production condition, but several factors have been suggested as causes for nephrocalcinosis. High ambient CO_2 levels have long been the primary suggested etiology for nephrocalcinosis although the published studies show contradictory results.

The main objective of this thesis was to provide better understanding of the prevalence and severity of nephrocalcinosis in farmed Atlantic salmon and to assess to which extend the condition is linked to environmental factors.

Field work was carried out at 11 salmon hatcheries and their receiving sea farms in Mid and Western Norway. 12 fish groups were sampled and the hatchery sampling consisted of collecting feed and water samples, in addition to kidney and gill tissue, and plasma from fish. In one of the 11 hatcheries one fish group was monitored for 6 months with monthly sampling of the same samples as the survey.

The results showed that nephrocalcinosis was observed in all of the hatcheries, but the prevalence varied greatly between hatcheries, ranging from less than 5% to 100% of the sampled fish. The mineral deposits were mainly identified as amorphous carbonate apatite (amCAP), which consist of calcium, phosphate and carbonate, and precipitate at pH > 6.8. We also found that the blood chemistry of fish with nephrocalcinosis was significantly different from fish without nephrocalcinosis. Pointing to disturbed physiology and homeostasis in fish with nephrocalcinosis.

Histopathology was the most precise tool for assessing nephrocalcinosis severity, but demands euthanasia. We therefore explored radiology as a non-invasive tool for detection of nephrocalcinosis. The results showed that amCAP was detectable with x-ray, although further optimization should be applied before the method can be used with high enough sensitivity and specificity. It would be beneficial to use radiology to test reversibility of nephrocalcinosis under controlled conditions. The method also shows potential as an initial investigation of nephrocalcinosis, with follow-up samples processed with histopathology, saving time, money and fish.

A multivariate analysis indicated that the most influencing factor for the prevalence of nephrocalcinosis was the supplementation of seawater during production. In the long-term monitoring we observed a time correlation between increased salinity and increased prevalence of nephrocalcinosis. The fish showed signs of long-term osmoregulatory stress with elevated levels of divalent ions in plasma. It has previously been suggested that electrolyte homeostasis, osmoregulation and mineral metabolism are closely related in salmonids, and from our data the most likely explanation for the link between SW supplementation and nephrocalcinosis was os-

moregulatory stress. The mechanism for calcium phosphate precipitation in the kidney is still not known, but may be linked to urine pH in Atlantic salmon combined with osmoregulatory stress.

Future studies on nephrocalcinosis should involve inducing osmoregulatory stress under controlled conditions, measurements of urine pH in Atlantic salmon acclimatized to both FW and SW, and exploring reversibility with radiology as diagnostic method.

Terminology

amCAP	Amorphous carbonate apatite
CaSR	Calcium sensing receptors
FT	Flow through aquaculture system
FW	Freshwater
GFR	Glomerular filtration rate
GH	Growth hormone
H&E staining	Haematoxylin and eosin staning
HSS	Haemorrhagic smolt syndrome
Hyperosmotic	Containing a greater concentration of solutes than another solution.
Hyposmotic	Containing a lesser concentration of solutes than another solution.
LD12:12	Light for 12 hours, darkness 12 hours
LD24:0	Continuous light for 24 hours
Osmoregulation	The process that regulates the osmotic pressure of fluids and electrolytic balance in organisms
Osmotic stress	Sudden change in solute concentration around a cell or organism
Osmoregulatory stress	Physiologic dysfunction caused by changes in the solute concentration around a cell or organism
PCA	Principal component analysis
PLS regression	Partial least square regression
PPT	parts per thousand
RAS	Recirculating aquaculture systems
RMSE	Root mean square error
SW	Seawater

CONTENTS

List of papers

I **C. Klykken**, A.K. Reed, A.S. Dalum, R.E. Olsen, M.K. Moe, K.J.K. Attramadal, L. Boissonnot (2022) Physiological changes observed in farmed Atlantic salmon (*Salmo salar* L.) with nephrocalcinosis, Aquaculture 554 (738104) https://doi.org/10.1016/j.aquaculture.2022.738104.

II **C. Klykken**, L. Boissonnot, A.K. Reed, P. Whatmore, K.J.K. Attramadal, R.E. Olsen. (2022) Gene expression responses in Atlantic salmon (*Salmo salar*) with severe nephrocalcinosis. Journal of Fish Diseases 00, 1– 14. https://doi.org/10.1111/jfd.13687

III **C. Klykken**, L. Boissonnot, A.K. Reed, K.J.K Attramadal, R.E. Olsen, A.S. Dalum. (2022) Radiological detection of nephrocalcinosis in farmed Atlantic salmon *Salmo salar* L.. Journal of Fish Diseases, 00, 1– 6. https://doi.org/10.1111/jfd.13704

IV **C. Klykken**, E.A. Khan, C. Karlsen, A.K. Reed, K.J.K. Attramadal, R.E. Olsen, L. Boissonnot (2023) Nephrocalcinosis in juvenile farmed Atlantic Salmon (*Salmo salar*) may be linked to osmoregulatory stress. Journal of Fish Diseases, 00, 1– 14. https://doi.org/10.1111/jfd.13815

Author contribution

I CK and LB were responsible for the study conception and design, and funding acquisition. CK carried out the field work, data analysis, and visualisation. MKM analysed the mineral deposits and AKR and ASD performed the histopathological assessments. CK wrote the manuscript with support from AKR, ASD and LB. LB, KJKA and REO supervised the work and aided in interpreting the results. All authors contributed in the review process.

II CK and LB were responsible for the study conception and design, and funding acquisition. CK carried out the field work, data analysis, and visualisation. AKR performed the histopathological assessments and PW preformed the RNA seq bioinformatics. CK wrote the manuscript with support from REO. LB , KJKA and REO supervised the work and aided in interpreting the results. All authors contributed in the review process.

III CK and LB were responsible for the study conception and design, and funding acquisition. CK carried out the field work, data analysis, and visualisation. AKR performed the histopathological assessments. CK wrote the manuscript with support from ASD. LB, KJKA and REO supervised the work and aided in interpreting the results. All authors contributed in the review process.

IV CK and LB were responsible for the study conception and design, and funding acquisition. CK carried out the field work, data analysis, and visualisation. EAK performed the qPCR analysis and CaK performed the statistical analysis and aided in data visualisation. AKR performed the histopathological assessments. CK wrote the manuscript with support from LB and CaK. KJKA and REO supervise the work and aided in interpreting the results. All authors contributed in the review process.

INTRODUCTION

General background

The salmon industry in Norway contribute to a yearly landing value of 8.6 billion EUR (Fiskeridirektoratet, 2021) and is one of the most important industries in rural Norway (Olaussen, 2018). In 2021, 412 million smolts were sold to sea facilities, while 135 million individuals either died or were destructed (Fiskeridirektoratet, 2021). The most common causes of mortality were reported by fish health personnel as non-infectious diseases like haemorrhagic smolt syndrome (HSS) and nephrocalcinosis (Sommerset et al., 2022). HSS and nephrocalcinosis were also reported as the current most important risk factors for production of Atlantic salmon smolts (Gåsnes et al., 2021).

Nephrocalcinosis was first described in Atlantic Salmon in 1999 (Fivelstad et al., 1999). The Norwegian Veterinary Institute reported the condition for the first time in 2006 in farmed salmon (Olsen et al., 2005) and nephrocalcinosis has been reported in every fish health report since then, both in recirculating aquaculture systems (RAS) and flow through systems (Gismervik et al., 2018; Sommerset et al., 2020; Hjeltnes et al., 2019; Sommerset et al., 2022; Jansen et al., 2021). To date, there is no systematic registration of nephrocalcinosis in aquaculture, but the condition has been more frequently reported by hatcheries, fish farms and fish health personnel in the last years.

Nephrocalcinosis

Nephrocalcinosis is described as deposits of mineral salts within kidney tubules and collecting ducts (Bruno, 1996). Mortality rates of farmed fish with nephrocalcinosis are generally low (Fivelstad et al., 1999; Nilsen et al., 2020) and the condition is considered to be reversible (Schlotfeldt, 1980; Fivelstad et al., 1999, 2003a; Nilsen et al., 2020). Despite this, the condition presents welfare challenges, as it is likely that impaired renal function results in a higher vulnerability to stress and disease. The condition is believed to develop during production on land (hatcheries), with a progressive aggravation until transfer to sea (Lazado et al., 2022; Loch et al., 2011).

Figure 1: Nephrocalcinosis in farmed Atlantic salmon. Left to right: increasing amounts of deposits. Photo credit: Aqua Kompetanse AS

Nephrocalcinosis can be visually identified in euthanized or dead fish (Bruno, 1996) (Fig.1), however, macroscopic assessment is not a precise tool, since small deposits are rarely visible to the naked eye (Klykken et al., 2020). To date, histopathology is therefore regarded as the standard in assessing the severity and progress of this condition. Observed changes involve basophilic mineral deposits that may progress into dilation of tubules and collecting ducts (Fig.2B), degeneration and necrosis of epithelium of the affected tubular structures (Fig.2C,D), which can lead to complete loss of epithelium with fibrosis of the basal membrane, and dilatation (Fig.2E), and further to complete loss of integrity of the wall, often accompanied by extensive tissue reactions in surrounding interstitial tissue (Fig.2F) (Fivelstad et al., 2018). Associated glomerular changes involve dilatation of the glomerular space, fibrosis and thickening of the parietal layer of the Bowman's capsule, and varying degree of per-glomerular fibrosis and glomerulitis (Fig.2G). The changes in the glomeruli are thought to be at least in part a result of urine stagnation (Docherty et al., 2006). In advanced cases, acute interstitial inflammation or chronic interstitial fibrosis is seen (Fig.2H), often in association with misshaped and degenerated tubuli (Fig.2H) and extensive dilatation of collecting ducts, with degeneration and necrosis of associated epithelium (Fig.2I). In addition to the excretory system, the kidney also consists of both hematopoietic, immunological, and endocrine tissues, all of which can be damaged upon the development of lesions into the interstitial compartment. These changes in the kidney tissue may lead to progressive amounts of renal damages (Sayer et al., 2004).

Deposits composition

Few studies have been conducted regarding the composition of kidney mineral deposits in farmed Atlantic salmon. To date, there is no peer-reviewed study on the composition of mineral deposits in salmon, but a master thesis from 2019 reported that the kidney deposits consisted of calcium and phosphate minerals (amorphous carbonate apatite, amCAP), carbonate apatite (CAP) and magnesium ammonium phosphate (struvite) (Thomsen, 2019). A survey conducted by Marin Helse AS on post-smolt salmon also reported that kidney deposits consisted of complexes of amCAP, with possible traces of complexes of magnesium, calcium and phosphate (whitlockite) (Sæther, 2019). This is in line with Béland et al. (2020), who stated that calcium phosphate and struvite calculi appears to be overrepresented in fish, such as rainbow trout *Oncorhynchus mykiss* (Gillespie and Evans, 1979; Smart et al., 1979; Bjerknes et al., 1994), wolffish *Anarhichas lupus* (Béland et al., 2020), and southern flounder *Paralichthys lethostigma* (Applegate et al., 2016), while Fİkrİ et al. (2000) found that rainbow trout had mineral deposits containing ammonium urate ($NH_4C_5H_3N_4O_3$) and calcium phosphate. Among other marine species, a study has been performed on cobia, (*Rachycentron canadum*), where the kidney stones consisted of pure calcium, oxalate and calcium phosphate (Klosterhoff et al., 2015).

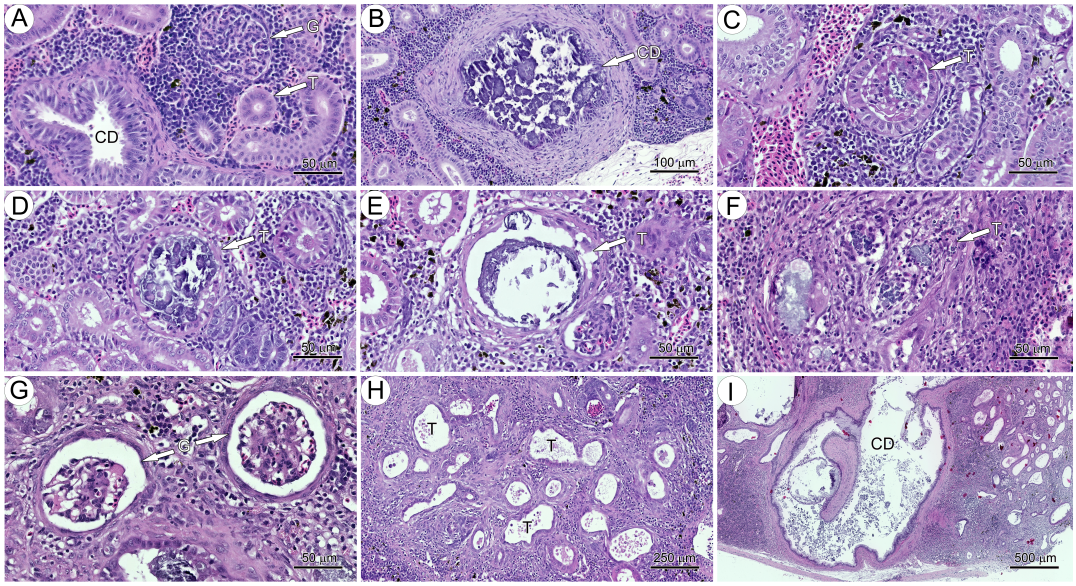


Figure 2: Histopathological changes associated with nephrocalcinosis. A) Normal tissue showing a healthy glomerulus *(G)*, tubulus *(T)* and collecting duct *(CD)*. B) Early lesion, with basophilic, amorphous deposits in a collecting duct. Note that the epithelium of the duct is intact, and that surrounding tissue appears without remarks. C) Early lesion in a tubule, with slight occurrence of degeneration and necrosis of the tubular epithelium. Deposits together with necrotic epithelial cells are seen in the tubular lumen. D) Moderate lesion in a tubule, with marked degeneration and necrosis of the epithelium and rich amounts of deposits in the lumen. E) Extensive, chronic lesions in a tubule, with complete loss of epithelium, fibrosis of the basal membrane, and dilatation of the lumen containing deposits. F) Advanced, chronic lesion with complete destruction of the tubular structure including the basal membrane, allowing the deposits getting in contact with the interstitial tissue inducing massive inflammation. G) Chronic changes seen in glomeruli, with dilatation of the Bowman's space, fibrosis and thickening of the parietal layer of the Bowman capsule, peri-glomerular fibrosis and moderate glomerulitis. H) Chronic changes, with degeneration of epithelium and dilatation of tubules, surrounded by extensive fibrosis replacing normal interstitial tissue. I) Chronic changes, with extensive dilatation of a collecting duct, degeneration and necrosis of epithelium and presence of deposits together with necrotic cells in the lumen. Photo credit: Pharmaq Analytiq AS

In humans and domestic (terrestrial) animals, the determination of kidney stone composition play an important role both in treatment and prevention (Tepeler and Turna, 2017; Kourambas et al., 2001; Koehler et al., 2009; Kravdal et al., 2015). It is therefore likely that the mineral composition of nephrocalcinosis in salmon could provide relevant information to prevent the condition.

Prevalence

To date, there is no systematic registration of nephrocalcinosis in salmon aquaculture, but the condition has been more frequently reported recently, but without clear indication of severity and etiology (Sommerset et al., 2020). In rainbow trout, nephrocalcinosis have been reported for several decades (Gillespie and Evans, 1979; Smart et al., 1979; Lall, 2010; Bjerknes et al., 1994; Mulcahy et al., 1983; Schlotfeldt, 1980; Fİkrİ et al., 2000; Myklebust, 2017; Hicks et al.,

1984; Harrison and Richards, 1979; Cowey et al., 1977; Knox et al., 1981; Loch et al., 2011; Cano et al., 2021; Raulic et al., 2021), both in experimental studies and in field and case studies in aquaculture.

Nephrocalcinosis has also been reported in other farmed aquatic species like European sea bass (*Dicentrarchus labrax* (Vandeputte et al., 2009; Mladineo et al., 2010), turbot (*Scophthalmus maximus*) (Saraiva et al., 2016), white sea bream (*Diplodus sargus* L.) (Golomazou et al., 2006; Gómez, 2000), Atlantic halibut (*Hippo-glossus hippoglossus*) (Jelmert et al., 1995), ballan wrasse (*Labrus bergylta*) (Cavrois-Rogacki et al., 2021), Nile tilapia (*Oreochromis niloticus*) (Chen et al., 2001), and sunfish (*Lepomis* sp.) Lohner et al. (2001). Mousavi et al. (2016), Gomez (2017), and Rahmati-Holasoo et al. (2020) also reported nephrocalcinosis in ornamental fish species; (discus (*Symphysodon discus*), angelfish (*Pterohyllum* sp.), goldfish (*Carassius auratus*), and oscar fish (*Astronotus ocellatus*) and Lewisch et al. (2013) observed nephrocalcinosis in longsnout seahorse (*Hippocampus reidi* Ginsburg). Nephrocalcinosis has also been detected in aquarium-housed and broodstock Atlantic wolffish, and spotted wolffish (*A. minor*) (Béland et al., 2020; Foss et al., 2003; François et al., 2021), and in aquarium-housed cobia (*Rachycentron canadum*) (Klosterhoff et al., 2015) and in showa koi (*Cyprinus carpio*) (Torpy et al., 2023).

Nephrocalcinosis seems to be rare in wild fish with only a few published reports of nephrocalcinosis in wild populations (flathead grey mullet, *Mugil cephalus* and freshwater mullet, *Myxus capensis* (McHugh et al., 2013)), white fish *Coregonus lavaretus* L., brown trout *Salmo trutta*, and charr *Salvelinus alpinus*; (Moiseenko and Kudryavtseva, 2001)). When reported in wild fish, the occurrence has been linked to reduced water quality. The same is true for farmed aquatic species, where it has been suggested that nephrocalcinosis is related to the suboptimal environmental conditions of intensive aquaculture (Gillespie and Evans, 1979; Smart et al., 1979; Bjerknes et al., 1994; Cavrois-Rogacki et al., 2021; Applegate et al., 2016; Béland et al., 2020; Klosterhoff et al., 2015; Lewisch et al., 2013; Loch et al., 2011).

Etiology

The etiology of nephrocalcinosis in fish is not known (Klosterhoff et al., 2015), but there is a consensus that the condition is related to intensive production conditions in aquaculture (Gillespie and Evans, 1979; Smart et al., 1979; Bjerknes et al., 1994; Cavrois-Rogacki et al., 2021; Applegate et al., 2016; Béland et al., 2020; Klosterhoff et al., 2015; Lewisch et al., 2013). Several studies on farmed Atlantic salmon indicated that both acute and long-term exposure to high levels of CO_2 in the ambient water can cause nephrocalcinosis (Fivelstad et al., 1999, 2003a, 2018; Hosfeld et al., 2008). The same results have been obtained in studies conducted with rainbow trout (Smart et al., 1979), Atlantic cod (*Gadus morhua*) (Damsgård et al., 2011), and spotted wolfish (Foss et al., 2003). In contrast, other comparable studies on elevated CO_2-levels did not find any signs of nephrocalcinosis (Fivelstad et al., 1998, 2007; Good et al., 2010, 2018; Mota et al., 2019; Martens et al., 2006; Graff et al., 2002). This may indicate that other environmental factors rather than in addition to suboptimal water CO_2-levels must play an important role in the development of nephrocalcinosis. For example high levels of phosphate together with high water pH have been suggested to cause nephrocalcinosis in longsnout seahorse (Lewisch et al., 2013) and in Nile tilapia reared in RAS where the prevalence of nephrocalcinosis was

reduced by changing the source of alkalinity from $CaCO_3$ to $NaHCO_3$ (Chen et al., 2001). Further pointing to suboptimal water quality, Bjerknes et al. (1994) reported that an abrupt change in water chemistry with sudden increased access to magnesium and calcium ions led to the development of nephrocalcinosis in farmed rainbow trout.

To the authors knowledge, no study has investigated the importance of feed composition on the development of nephrocalcinosis in Atlantic salmon, but several studies have been conducted in rainbow trout. It has been suggested that magnesium deficiency (Knox et al., 1981), selenium toxicity (Hicks et al., 1984; Hilton and Hodson, 1983), toxic levels of disodium arsenate heptahydrate (Cockell et al., 1991) and high content of phosphorus and calcium (Knox et al., 1981; Cowey et al., 1977) in feeds can be linked to nephrocalcinosis in rainbow trout. It has also been suggested that high ratios of calcium and/or phosphorus to magnesium in feed can cause nephrocalcinosis in chinook salmon (*Oncorhynchus tshawytscha*)(Richardson et al., 1985).

A high number of scientific papers have reported that unsuitable nutrients are the most important factor in the development of kidney stones in mammals (Tion et al., 2015). These include unbalanced dietary concentrations of vitamin D, calcium, phosphorus, or acid (Shavit et al., 2015; Phillips et al., 1986; Gambaro and Trinchieri, 2016). It has also been suggested that an adapted diet can reverse the condition (Davies, 2016). It is therefore possible that the commercial feeds used in the aquaculture industry have a suboptimal nutrient composition with regard to kidney stone formation and that adaptations could be made to help prevent the development of nephrocalcinosis.

The teleost kidney

The kidney of teleosts is mesonephric and retains a segmental structure (Bone and Moore, 2008), but lack a well defined cortex, medulla, and loop of Henle (Engelund and Madsen, 2011). In the anterior part of the kidney, the vasculature, glomeruli, and tubular segments are interwoven and interspersed with hematopoietic tissue (Anderson and Loewen, 1975; Resende et al., 2010) and the teleost kidney differs from the mammalian kidney in both number and size of glomeruli as well as differentiation of functional segments of nephrons (Engelund and Madsen, 2011). The nephrons in the teleost kidney are divided into sections: glomerulus, proximal tubule I and II, intermediate segment, the distal tubule, and the collecting duct (Hickman and Cleveland, 1968). The anatomical and regulatory properties of the different sections may differ depending on whether the fish is adapted to FW or SW (Takvam et al., 2021).

Osmoregulation

Osmoregulation describes the processes that enables a fish to maintain internal homeostasis through maintaining cellular fluid composition and volume (Evans, 2011). Osmoregulation is a fundamental process since fish remain in continuous contact with their environment, constantly challenged to maintain plasma ion concentrations within the defined range necessary for proper cellular function (Evans, 2010). There are three primary organs involved in osmoregulation in fish; kidney, gills and intestine (Talbot et al., 1992; Grosell, 2010; Varsamos et al., 2005). In FW the fish is hyperosmotic, with a continuous osmotic influx of water and a diffusive loss

of ions through the gills and skin. To maintain fluid and electrolyte homeostasis, Na^+ and Cl^- are actively pumped into the blood over the gills (Evans, 2011), while the kidney filter large amounts of blood in the glomeruli to excrete large volumes of diluted urine (Hickman and Cleveland, 1968; Beyenbach, 2004).

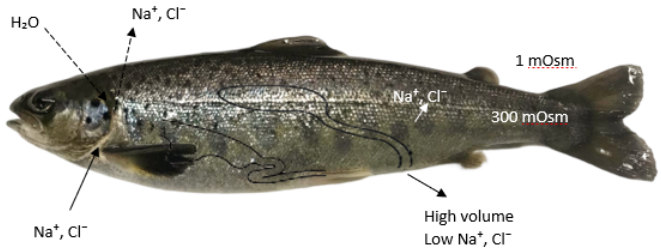


Figure 3: Osmoregulation in FW teleosts. Adapted from Evans (2008). Photo credit: Christine Klykken (Aqua Kompetanse AS)

In SW the fish is hypoosmotic, with constant loss of water to the environment by osmosis over the gills and skin. The fish counteracts the loss by drinking seawater, and thereby incur additional Na^+ and Cl^- loading which accompanies this enteric water absorption (Marshall and Grosell, 2005). The ambient water is three times more concentrated than blood, and by the time the remaining fluid reaches the distal part of the intestine, the volume has been greatly reduced (Wilson, 2011). The chemistry of the fluid has also changed from the original ingested seawater to instead being dominated by divalent ions and HCO_3^- (Wilson, 2011). This excess in divalent ions is excreted over the kidney.

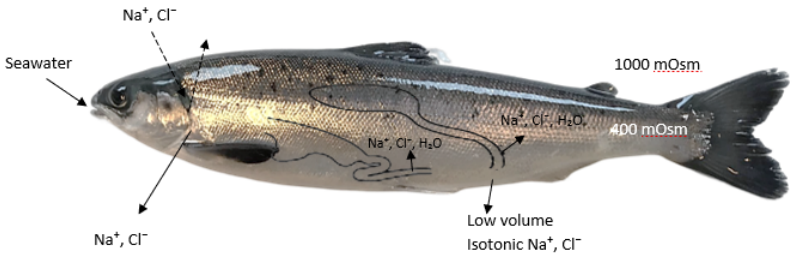


Figure 4: Osmoregulation in SW teleosts. Adapted from Evans (2008). Photo credit: Christine Klykken (Aqua Kompetanse AS)

Approximately 3-5% of teleosts are euryhaline (Evans, 1984) with the ability to make major adjustments in renal function as the salinity changes (Beyenbach, 2004). Most scientific papers on renal function in fish have focused on either FW or SW acclimated fishes (Takvam et al., 2021). In the hyper-osmotic environment of FW, one of the main tasks of the kidney is to excrete excess water while reabsorbing solutes (McDonald, 2019; Hickman and Cleveland,

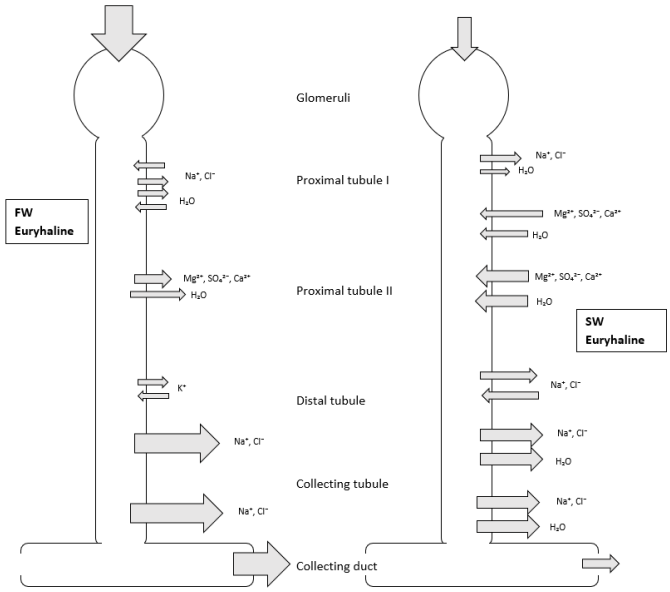


Figure 5: Overview of nephron segments in freshwater teleost (left; A) and saltwater teleost (right; B) with water and ion movement adapted from Takvam et al. (2021). A) High GFR followed by reabsorption of monovalent ions and H_2O in the proximal tubule I. In proximal tubule II H_2O and divalent ions are reabsorbed. In the distal and collecting tubules monovalent ions are reabsorbed and high volume of diluted urine is passed through the collecting ducts. B) Low GFR followed by reabsorption of monovalent ions and H_2O in the proximal tubule I. In proximal tubule II secretion of Na^+, Cl^-, Mg^{2+}, $SO_4{}^{2-}$, H_2O in the early proximal tubule. Reabsorption of glucose, other organic solutes, Na^+, Cl^- and H_2O in the late proximal tubule. Reabsorption of Na^+, Cl^- and H_2O in the urinary bladder.

1968). In FW, water is mainly absorbed in the proximal tubule along with some monovalent ions, while the distal segment of the nephron is the primary site for monovalent ion absorption (Bone and Moore, 2008) (Fig 5 left).

In SW, the fish is threatened by dehydration and therefore drink ambient water containing large concentrations of divalent ions. The primary function of the kidney in SW is therefore to excrete divalent ions in strongly reduced isotonic urine (Beyenbach, 2000; Bone and Moore, 2008; Takvam et al., 2021). In the marine teleost this is obtained by reducing the GFR and secretion of monovalent ions in the proximal tubule, driven by water excretion (Bone and Moore, 2008). Divalent ions, in particular Mg^{2+} and $SO_4{}^{2-}$, are secreted into the proximal tubule while the distal tubule mainly reabsorbs water (Bone and Moore, 2008) (Fig. 5 right).

A change in salinity of the surrounding environment of euryhaline teleosts, like Atlantic salmon transferred from FW to SW, therefore requires that the kidney undergoes a major functional switch from being filtrating in FW to being predominantly secretory in SW conditions (Madsen et al., 2020).

Parr-Smolt transformation/Smoltification

In wild Atlantic salmon the parr-smolt transformation or smoltification is the process were stream-dwelling parr changes to the downstream migrating smolt (Boeuf, 1993; McCormick et al., 1997; McCormick, 2012). During parr-smolt transformation there appears to be a link between altered behaviour and physiological changes, and increased salinity tolerance is one of the most important changes caused by alteration in the function of the major osmoregulatory organs (McCormick, 2012). The parr-smolt transformation involves several endocrinologically mediated changes affecting behaviour, morphology, and physiology (Strand et al., 2007) and several endocrine signalling systems are involved (Björnsson et al., 2011). The most important hormones involved are growth hormone (GH), cortisol, prolactin and thyroid hormones (Morera et al., 2021).

The timing of smoltification is regulated by seasonal changes in environmental cues (day length and water temperature) and a body size threshold. Atlantic salmon need to reach a threshold size of 12-13 cm in order to respond to the photoperiod cue of increased day length (spring) (McCormick et al., 2007). This size threshold is normally reached in 1-8 years, depending on the growth rate in the pre-smolt stages (Heggberget et al., 1992; Metcalfe and Thorpe, 1990). It is also well established that photoperiod plays the key role in triggering smoltification (McCormick et al., 1987; Duston and Saunders, 1990) and that GH appears to be the first hormone to increase following increased day length (McCormick, 2012). Increased water temperatures rather appears to determine the rate of changes in smolt characteristics (McCormick et al., 1997). For many smolts the initiation of migration will result in SW entry within days or weeks (McCormick, 2012).

In commercial hatcheries, Atlantic salmon are normally cultured in constant light (LD24:0) from first feeding until smoltification is induced by introducing an artificial winter signal (light for 12 hours and darkness for 12 hours; LD12:12) followed by LD24:0 (Martinez et al., 2021). This change of photoperiod mimics the seasonal change from winter to spring, and the commercial parr interprets this change of photoperiod as a signal to prepare for migration (entry into seawater). The change of photoperiod, induces an increase in gill mRNA expression of Na^+, K^+-ATPase α1b isoform and a reduction in the expression of the α1a isform (Nilsen et al., 2007), reflecting development of hypoosmoregulatory ability and seawater tolerance necessary for successful smoltification (Handeland et al., 2003; McCormick et al., 1987; Stefansson et al., 1991). When the hypoosmoregulatory capacity of the salmon is sufficiently high (Mortensen and Damsgård, 1998), the smolts are considered to be in the "smolt-window", a relatively short period when they can be successfully transferred from freshwater to seawater (Lundqvist and Eriksson, 1985). The "smolt-window" is shorter at high water temperatures (Handeland et al., 2014) and if the fish is not transferred to SW within the "smolt-window" the hatchery reared smolts lose their migratory urge, salinity tolerance, and the underlying osmoregulatory changes (Duston and Saunders, 1990; Hoar, 1988). The endocrine control of the loss of smolt characteristics also called desmoltification (Björnsson et al., 2011) has not been fully elucidated yet, but it is not a synchronised process, nor is it a parr-reversion (Stefansson et al., 1998).

One of the main differences between wild salmon and commercially reared salmon is the age for onset of smoltification. Wild Atlantic salmon can stay in FW for several years before undergoing smoltification (Heggberget et al., 1992), while commercially reared salmon usually undergo

SW adaption in 8-16 months (Mørkøre and Rørvik, 2001). This is connected to the year round demand for market-size fish and has resulted in different strategies for smoltification in order to have stable year-round production (Duston and Saunders, 1995).

The traditional "light stimulated smoltification", described above, has previously been shown to reduce appetite and growth and the fish farming industry has therefore explored other methods for smoltification including a dietary treatment concept ("dietary stimulated smoltification") (Striberny et al., 2021) and exposure to gradually increasing salinity (Lysfjord et al., 2004). "Dietary stimulated smoltification" involves a salt/ion and tryptophan supplemented feed, usually introduced to the fish late in the FW production phase and provided until transfer to sea (Striberny et al., 2021). In contrast to standard commercial feed with a NaCl content of 1-4%, the diet used to stimulate smoltification ("salt feed") usually consist of 6-8% Na^+ and Cl^- (Bucking et al., 2013; Salman, 2009). The mechanisms for salinity tolerance obtained through the diet, may include signalling through G protein-coupled calcium-sensitive receptors (CaSR) as well as ion- and metabotropic glutaminergic receptors (Nearing et al., 2002). CaSR has also been shown to be activated by a range of different amino acids, including tryptophan (Loretz, 2008). "Salt feed" has also been shown to have beneficial effects on growth and survival post SW transfer for salmonids (Wood and Bucking, 2011; Kristensen et al., 2012).

A notable number of commercial hatcheries use exposure to gradually increasing salinity as their smoltification protocol (Toften, 2011). However, there is no scientific literature describing this method for parr-smolt transformation, but gradual transfer to SW is likely to promote osmoregulatory adaptation and SW tolerance in salmonids (Lee et al., 2022). Still, a survey conducted among smolt producers in Norway, Great Britain, the Faroe Islands, Canada and Chile, show that the majority of producers use a combination of the above described methods for smoltification including temperature changes (Ytrestøyl, 2022).

A large proportion of farmed Atlantic salmon in Norway today are reared in RAS with water temperatures of 12-14° almost throughout the whole production on land (Ytrestøyl, 2022). Combined with continuous light, this gives a rapid growth in the early life stages and results in a high weight when transferred to sea (Ytrestøyl, 2022). Production of large smolts ($>$ 200 g) demands a longer production time on land, which can be obtained either by keeping the smolt in brackish water concentrations or by increasing the length of the "winter signal" for up to several months (Ytrestøyl, 2022). There is substantial scientific evidence stating that a winter signal is necessary for obtaining good seawater tolerance in small smolts, but there is little available information on how the absence of a winter signal affects the performance of large smolt transferred to sea (Striberny et al., 2021; Ytrestøyl et al., 2022).

THESIS OBJECTIVES

The main aim of this thesis was to provide a better understanding of the prevalence and severity of nephrocalcinosis in farmed Atlantic salmon and to assess to which extend the condition was linked to environmental factors.

Specific aims:

I Document the prevalence and severity of nephrocalcinosis in farmed Atlantic salmon and describe physiological changes in fish with nephrocalcinosis.

II Study the effects of severe nephrocalcinosis on fish physiology and gene expression patterns, in order to provide new insight into the mechanisms of the disease.

III Explore methods to detect and evaluate the severity of nephrocalcinosis.

IV Study the development of nephrocalcinosis on group level in a commercial facility to identify critical phases in production linked to increased risk of developing nephrocalcinosis including environmental factors.

SUMMARY OF PAPERS

Paper I

Physiological changes observed in farmed Atlantic salmon (*Salmo salar* L.) with nephrocalcinosis.

Authors: Christine Klykken, Anne K. Reed, Alf S. Dalum, Rolf E. Olsen, Morten K. Moe, Kari J.K. Attramadal and Lauris Boissonnot.

Aquaculture 2022, https://doi.org/10.1016/j.aquaculture.2022.738104

In this paper, we studied the prevalence and severity of nephrocalcinosis in farmed Atlantic salmon in Mid-Norway. Data was collected from 14 fish groups in twelve different hatcheries, both flow through (FT) and re-circulating aquaculture systems (RAS), and their receiving sea farms.

Nephrocalcinosis was observed in all the hatcheries in this study, but the prevalence varied greatly between hatcheries, ranging from less than 5% to 100% of the sampled fish. The total proportion of fish with nephrocalcinosis was 45% in the hatcheries. Among fish that were sampled one month after sea transfer, the prevalence of nephrocalcinosis had decreased to 18%. The mineral deposits were mainly identified as amorphous carbonate apatite (amCAP), which consist of calcium, phosphate and carbonate. The blood chemistry of fish with nephrocalcinosis was significantly different from fish without nephrocalcinosis. The most noteworthy differences were the levels of calcium, phosphate, magnesium and apartate aminotransferase (AST). These were elevated in fish with nephrocalcinosis indicating disturbed ion regulation and tissue damage.

This study clearly showed that nephrocalcinosis is a common production related disorder in farmed Atlantic salmon in hatcheries in Mid-Norway. The fish studied exhibited a broad range of severities, ranging from sparse amounts of deposits in tubules and collecting ducts to pathological changes in glomeruli and the interstitial tissue. Although the majority of the examined fish were only mildly affected, our results revealed that nephrocalcinosis is a welfare challenge. A considerable percentage of the fish affected by nephrocalcinosis had altered plasma chem-

istry, indicating stress and osmoregulatory disorders likely connected to environmental factors including suboptimal water quality.

Paper II

Gene expression responses in Atlantic salmon (*Salmo salar*) with severe nephrocalcinosis.

Authors: Christine Klykken, Lauris Boissonnot, Anne K. Reed, Paul Whatmore, Kari J.K. Attramadal and Rolf E. Olsen.

Journal of Fish Diseases 2022, https://doi.org/10.1111/jfd.13687

This is the first transcriptome study profiling the response of Atlantic salmon (*Salmo salar*) with chronic and severe nephrocalcinosis. The study showed numerous genes and KEGG pathways being differentially expressed in salmon with nephrocalcinosis compared with healthy salmon (Ctrl). The transcriptome profiles of salmon affected by severe nephrocalcinosis revealed an upregulation of inflammation and repair processes together with a massive shutdown of metabolism. These responses were most likely related to the severe tissue damage observed on histology resulting in kidney failure. The results were supported by changes in blood chemistry of fish with severe nephrocalcinosis. Taken together, severe nephrocalcinosis is a major welfare challenge that likely makes the salmon more sensitive to additional stressors. The reduced renal function may have fatal consequences for the fish both in the hatchery and when transferred to sea.

Paper III

Radiological detection of nephrocalcinosis in farmed Atlantic salmon *Salmo salar* L.

Authors: Christine Klykken, Lauris Boissonnot, Anne K. Reed, Kari J.K. Attramadal, Rolf E. Olsen and Alf S. Dalum.

Journal of Fish Diseases 2022, https://doi.org/10.1111/jfd.13704

Research on nephrocalcinosis has been greatly hampered by the lack of non-invasive methods to assess the condition. In this paper we explored radiology as a diagnostic tool for nephrocalcinosis. A total of 80 farmed Atlantic salmon (mean weight 198 g) were sampled from two RAS facilities, randomly sampled among visually healthy individuals. Left anterior lateral view radiographs were taken and samples of whole kidney were possessed for histopathological analysis.

The distribution and density of mineral deposits were clearly observed on the x-ray images. The sensitivity of radiology for nephrocalcinosis was high, but the correlation between the total histology score and the radiological assessment was significant but low.

Our results show that the method is suitable for the detection of mineral deposits characteristic for nephrocalcinosis. We suggested a scoring model that can be used for evaluating the severity of nephrocalcinosis on radiographs. The sensitivity and specificity of the method should be

further increased with modification of the scoring model. Radiology will make it possible to investigate nephrocalcinosis on individual and group level over time and thus enable testing the effect of various measures to prevent the development of the disease and possibly understanding the etiology.

Paper IV

Nephrocalcinosis in juvenile farmed Atlantic salmon (*Salmo salar*) may be linked to osmoregulatory stress.

Authors: Christine Klykken, Essa A. Khan, Camilla Karlsen, Anne K. Reed, Kari J.K. Attramadal, Rolf E. Olsen and Lauris Boissonnot.

Journal of Fish Diseases 2023, https://doi.org/10.1016/j.aquaculture.2022.738104

There is no consensus on the etiology of nephrocalcinosis. This makes it problematic to implement proper measures to limit its development. We performed a survey of the prevalence of nephrocalcinosis as well as environmental factors in eleven different fish groups in Mid-Norway and a 6-month study in one of the hatcheries.

A multivariate analysis indicated that the most influencing factor for the prevalence of nephrocalcinosis was the supplementation of seawater during production. In the long-term monitoring we observed a time correlation between increased salinity and increased prevalence of nephrocalcinosis. Salinity fluctuations can cause osmotic stress, and result in unbalanced levels of ions in the fish blood. This was clearly demonstrated in our study, as the fish experienced chronic hypercalcemia and hypermagnesemia. Both magnesium and calcium are excreted over the kidney and it is possible that their elevated levels in plasma resulted in an mineral overload in the kidney. This could have in turn caused the aggregation of calcium deposits.

The results of this study point to a causal relationship between osmotic stress induced by salinity changes and/or a challenging level of salinity combined with the fish developmental stage and previous history, and the development of nephrocalcinosis. Still, fluctuating salinity is not the only factor that can cause osmotic stress in salmon. For example, high levels of CO_2 can induce gill lesions (Fivelstad and Binde, 1994), which affect the osmoregulatory capacity of individuals. It is therefore possible that the different parameters indicated by previous studies as causative factors for nephrocalcinosis may all have induced osmotic stress that in turn triggered mineralization in kidneys.

STUDY DESIGN

Nephrocalcinosis survey

Field work was carried out at 11 salmon hatcheries and their receiving sea farms in Mid and Western Norway (Fig.6). 12 fish groups were sampled and the hatchery sampling consisted of collecting feed (Tab.4) and water samples (Tab.8, Tab.5), in addition to kidney and gill tissue, and plasma from fish. Fish were randomly sampled among visually healthy individuals (with normal swimming behaviour, absence of external injuries/lesions, and no sign of emaciation) and the fish were not starved before sampling. Apart from nephrocalcinosis and haemorrhagic smolt syndrome (HSS), no health issues were observed or reported for the fish groups.

Due to interest from a farmer outside the original industrial partner group in addition to an new fish group from one of the partners, data was analysed on two additional fish groups and included in **Paper I**. Unfortunately due to incomplete data set for the environmental factors these had to be excluded from **Paper IV**.

The histopathological diagnosis of nephrocalcinosis was defined as the presence of amorphous (structureless), basophilic deposits in tubules, collecting ducts and excretory ducts. Histopathological sections were analysed, and presence of deposit and degree of tissue damage was evaluated and given a score based on the type and distribution of changes. The nephrocalcinosis score defines and categorises the presence of deposits, degree of tissue damage in structures with deposits, glomerular alterations and pathology in the interstitial tissue. Pathological changes were divided into 4 sub-categories. Each category was weighted by the effect the different pathological changes are believed to have on the development of the condition and the time it will take to heal.

In order to explore possible mechanisms behind nephrocalcinosis, transcriptome profiling was utilised on contrast groups (severe nephrocalcinosis and no nephrocalcinosis). At the time of analysis fish with severe nephrocalcinosis were only found in one hatchery. Among the sampled fish from this hatchery non were diagnosed as healthy. Healthy fish were therefore selected from a different hatchery based on the following criteria; same production technology and similar size. Previous studies conducted by the co-authors of **Paper II** has shown that 8 samples in

Figure 6: Sampling area

each group were sufficient to detect differences in gene expression patterns for contrast groups, and were therefore chosen as n in this study.

Long-term monitoring of nephrocalcinosis development

A long-term study was performed in one of the 11 hatcheries studied. This hatchery was chosen based on having historically moderate to high prevalence of nephrocalcinosis. The fish group was monitored for 6 months until transfer to sea, with monthly sampling. The sampling consisted of kidney and gill tissue, and plasma from randomly selected fish (visually healthy). Water quality was routinely monitored by the staff, and the data was provided for this project in addition to data on rearing conditions. Fish with HSS were excluded from further analysis.

Pilot study - radiological detection of nephrocalcinosis

80 smolts were sampled from two different RAS facilities (hatcheries) among healthy looking individuals. Left anterior lateral view radiographs were taken of all fish using a standard portable x-ray unit. Whole kidney were sampled for histological analysis of nephrocalcinosis from all individuals.

MAIN RESULTS

I **Nephrocalcinosis is a widespread disorder in farmed Atlantic salmon.**

Among the sampled fish groups, nephrocalcinosis were diagnosed in all groups. The prevalence and severity of the disorder varied extensively between hatcheries with most of the fish (68%) displaying mild forms of nephrocalcinosis, exhibiting at most, negligible tissue damage.

II **Fish with nephrocalcinosis display disturbed physiology compared to fish without nephrocalcinosis.**

A considerable percentage of the fish affected by nephrocalcinosis had altered plasma chemistry, indicating increased stress levels and disturbed osmoregulation. It was not possible to diagnose nephrocalcinosis with a blood sample at this stage, but elevated plasma levels of magnesium, calcium, glucose and aspartate aminotransferase (AST) could be used as indicators for disturbed physiology that may be related to nephrocalcinosis.

III **The mineral deposits characteristic for nephrocalcinosis consists mainly of amorphous carbonate apatite (amCAP) and are detectable with x-ray.**

Nephrocalcinosis in Atlantic salmon was mainly identified as a calcium dominated mineral. The distribution and density of calcium mineral deposits were clearly observed on x-ray images and offers promising prospects for the development of a non-invasive assessment of nephrocalcinosis in fish.

IV **Osmoregulatory stress is a risk factor for nephrocalcinosis.**

A multivariate analysis of environmental factors and nephrocalcinosis in several hatcheries indicated that the most influencing factor for the prevalence of nephrocalcinosis was the supplementation of seawater during smolt production. The results were backed by a correlation between increased salinity and nephrocalcinosis prevalence observed during a long term monitoring in one hatchery. The fish in this hatchery experienced osmoregulatory stress evident by chronic plasma hypercalcemia and hypermagnesemia prior to the increased prevalence of nephrocalcinosis pointing to osmoregulatory stress being a trigger for nephrocalcinosis.

GENERAL DISCUSSION

Diagnostic methods for detecting nephrocalcinosis

There are several methods for detection of nephrocalcinosis including radiology, computed tomography, sonography, histology (Manz et al., 1980) and macroscopic assessment. The most common methods for detection in salmonids are macroscopic assessment (Heinen et al., 1993; Lazado et al., 2022; Smart et al., 1979) and histopathology (Smart et al., 1979; Fivelstad et al., 2018; Mota et al., 2019).

Large amounts of deposits in the kidney are visible to the naked eye (Fig.1, **Paper I, II**). There is however no standardised macroscopic scoring of nephrocalcinosis in Norway, but several fish health services have developed their own protocols (Sæther, 2019). One of these protocols was used in the nephrocalcinosis survey to score nephrocalcinosis from no deposits (score 0) to extensive amounts of deposits with swellings of the kidney tissue (score 4) (Fig.7).

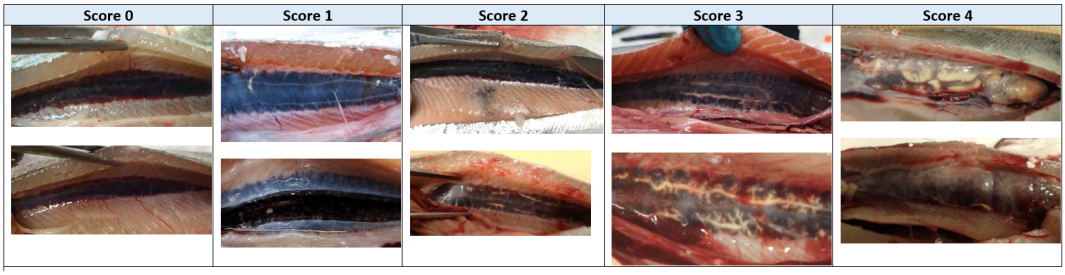


Figure 7: Macroscopic scoring of nephrocalcinosis used in the PhD-project. Photo credit: Aqua Kompetanse AS and Marine Helse AS.

Kidney tissue from all fish were also assessed with histology (**Paper I**, Fig.2) and the correlation between the macroscopic scoring and histopathological assessment was poor (Fig.8) (Klykken et al., 2020). The same was also the case in a screening program in Ireland in the 1980's (Mulcahy et al., 1983). One reason for this might be that small amounts of deposits are both difficult and sometimes impossible to detect macroscopically.

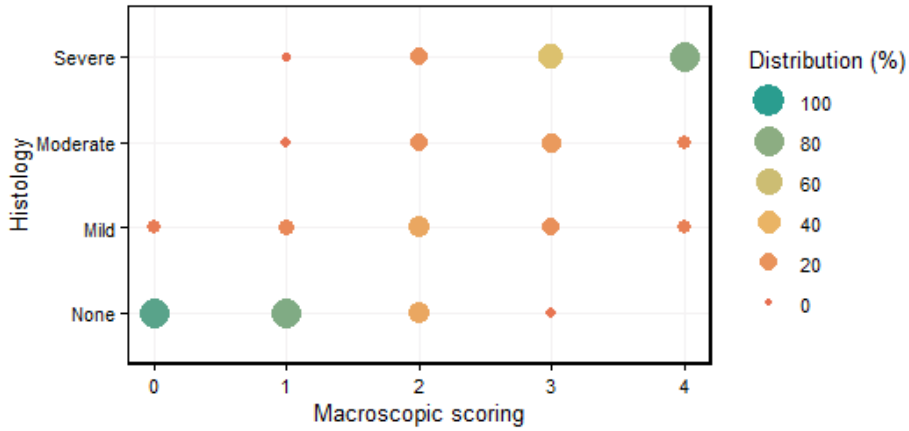
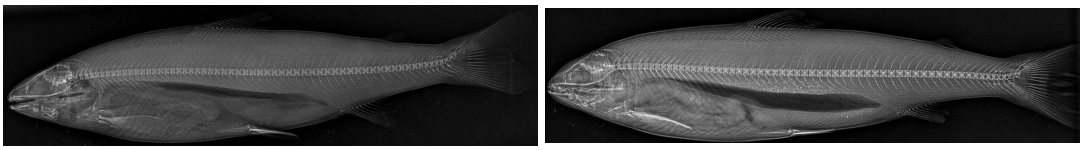


Figure 8: Macroscopic scoring of nephrocalcinosis compared with histopathological assessment. Size of circle indicates percentage assessment which is similar for visual and histopathological assessment.

It is also possible that the poor correlation between macroscopic scoring and histopathology can be related to the fact that the amount of tissue damage and the amount of deposits found in the histopathological sections do not always correlate (**Paper I**). For this reason one should be cautious when using macroscopic scoring, since both under and over diagnosis of nephrocalcinosis may occur.

Although histopathology is a good diagnostic tool for nephrocalcinosis, one of the major draw backs is the need for euthanasia. There are several reasons why this is a disadvantage; first there is the ethical aspect of unnecessary euthanasia of fish, secondly there is the economical aspect for the fish farmer, and thirdly it makes it impossible to study the disease over time on an individual level.

Nephrocalcinosis is believed to be, at least in mild cases, reversible (Schlotfeldt, 1980; Fivelstad et al., 1999, 2003a). Without non-lethal methods, reversibility on a individual level is not possible to study and will remain as an untested hypothesis. One possible non-lethal method for detecting mineral deposits in the kidney is radiology. We did a pilot study to confirm that mineral deposits characteristic for nephrocalcinosis were detectable in Atlantic salmon (**Paper III**, Fig.9).



(a) **(b)**

Figure 9: Radiographs of Atlantic salmon smolts without (a) and with (b) nephrocalcinosis. Photo credit: Aqua Kompetanse AS.

The major advantages of using portable radiography is that it allows for a fast and cost-effective diagnosis of mineral deposits characteristic for nephrocalcinosis that can be performed on a large number of sedated fish at the fish farming sites. Compared to histopathology this will allow for a larger sample size resulting in a greater accuracy when assessing the prevalence. Still, there are limitations to radiology, since soft tissue changes, not including mineralization, cannot be seen on radiographs. In general, it will be impossible to evaluate the degree of soft tissue lesions associated with deposits based on radiology alone. Another major limitation is the lack of possibility to differentiate between different types of pathologies associated with mineral deposits in kidney tissue, meaning that conditions such as chronic granulomatous inflammation with secondary central calcification cannot be differentiated from deposits due to nephrocalcinosis (**Paper III**). It would therefore be both cost- and time-effective, and professionally sound to implement a step-wise strategy in diagnosing nephrocalcinosis as suggested in pediatric medicine (Manz et al., 1980), where if nephrocalcinosis is suspected, radiology should be the first investigation followed by histology if the radiograph is negative or there is a discrepancy between the clinical data and the radiograph.

Nephrocalcinosis - prevalence, severity and welfare implications

Nephrocalcinosis is a widespread production disorder in juvenile farmed Atlantic salmon and there are large variations in prevalence between commercial hatcheries in Norway (**Paper I**, Fig.10a). No link between production technology (RAS and FT) has been observed (**Paper I**), which show that nephrocalcinosis may arise and progress in different environments. To the authors knowledge, nephrocalcinosis in wild Atlantic salmon has to date not been reported, implying that the disease is directly linked to the intensive production conditions in aquaculture (Gillespie and Evans, 1979; Smart et al., 1979; Bjerknes et al., 1994; Cavrois-Rogacki et al., 2021; Applegate et al., 2016; Béland et al., 2020; Klosterhoff et al., 2015; Lewisch et al., 2013).

Nephrocalcinosis is a chronic condition (Smart et al., 1979) which can aggravate over time. Histopathological assessment of changes in kidney tissue associated with nephrocalcinosis, reveals how the disease may progress (scoring model, **Paper I**). Initially amorphous calcium apatite will precipitate in the tubulus, collecting ducts and/or urinary bladder giving small to negligible changes in the epithelium of the tubular structures (categorized as mild changes in the nephrocalcinosis score, **Paper I**). With time, the mineral deposits will aggravate and lead to increased damage to the tubular walls and eventually result in complete loss of epithelium with fibrosis of the basal membrane (categorized as moderate to severe changes in the nephrocalcinosis score, **Paper I**). Finally, the changes in the kidney tissue will result in complete loss of integrity of the wall, often accompanied by extensive tissue reactions (inflammation) in surrounding interstitial tissue (categorized as severe changes in the nephrocalcinosis score, **Paper I, II**). We would expect that the degree of severity of nephrocalcinosis affects the overall health and welfare of the salmon, as well as the performance and survival.

Atlantic salmon with severe nephrocalcinosis have a different blood chemistry profile compared to salmon without nephrocalcinosis (**Paper I, II**). Specifically the electrolytes calcium, magnesium and phosphate were significantly elevated in fish with severe nephrocalcinosis indicating

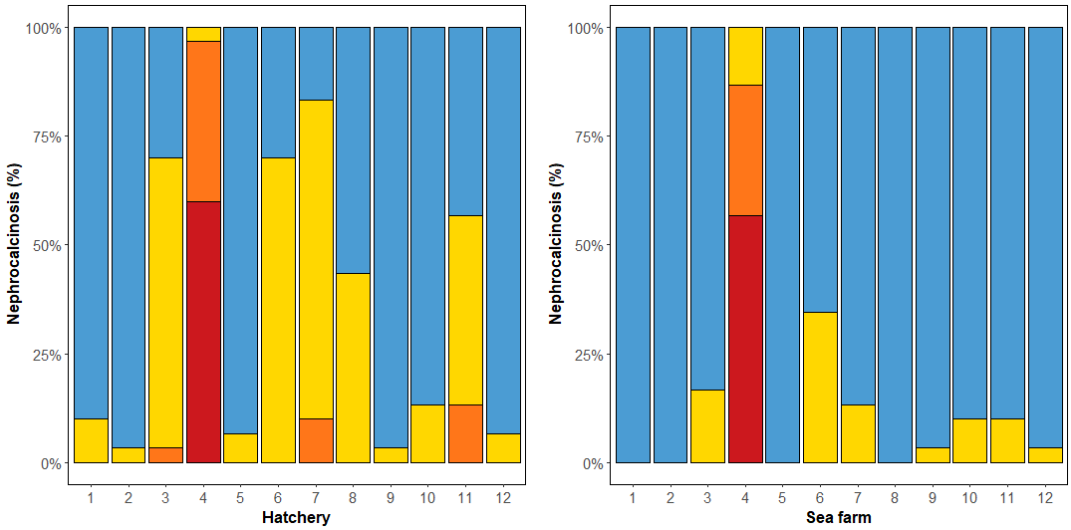


Figure 10: Nephrocalcinosis in a) hatcheries in Mid Norway and b) receiving sea farm. Blue colour indicates no nephrocalcinosis, yellow indicates mild changes, orange shows moderate changes and red indicates severe changes in the kidney tissue as found from histopathological examination (n=30 for each facility) (**Paper I, IV**).

disturbed osmoregulation. The seemingly failure to osmoregulate properly is undoubtedly connected to kidney malfunction, since the kidney is the primary site for divalent ion excretion in fish (Beyenbach, 2000; Bone and Moore, 2008; Takvam et al., 2021). Further supporting this notion are the observed fibrosis, necrosis, and tissue reactions in the kidney (**Paper II**), and the elevated levels of plasma AST which is regarded as a good indicator for tissue damage in fish (Peres et al., 2015; Wagner and Congleton, 2004; Li et al., 2011). Gene expression analysis of fish with severe nephrocalcinosis also revealed massive downregulation of metabolism and energy production, upregulation of signalling pathways important for tissue repair and function maintenance, and upregulation of inflammatory responses (**Paper II**) which can all be linked to extensive tissue damage. In general, fish groups with high prevalence of severe degrees of nephrocalcinosis are less robust to handling (stressful events) and sea transfer is likely to result in increased mortality (pers. observation).

In other fish species, nephrocalcinosis has been associated with decreased growth, impaired renal function (Lall, 2010), and mortalities when combined with stressful events (Wedemeyer, 1996; Jelmert et al., 1995), but mortality rates in salmon with nephrocalcinosis have previously been described as low (Fivelstad et al., 1999; Nilsen et al., 2020). The reason for this may be that the majority of farmed salmon display mild degrees of nephrocalcinosis (**Paper I**, Fig.10) and mild degrees of nephrocalcinosis is limited to amorphous mineral deposits in collecting ducts and tubulus, with only minor changes in the epithelium of the tubular structures affecting between 10% and 50% of the excretory system (**Paper I**). It is possible that fish, Atlantic salmon in particular, can obtain sufficient renal function with reduced amount of functioning

tubules. In the comparable species rainbow trout, Brown et al. (1980) found that 45% of the tubules were filtrating in FW and only 5% were filtrating in SW.

Although mild degrees of nephrocalcinosis don't seem to cause direct mortality, the mild and subclinical changes in the kidney may alter metabolism and physiology. In fish with mild degrees of nephrocalcinosis plasma levels of different enzymes, metabolites and electrolytes differed from fish without nephrocalcinosis (**Paper I**).

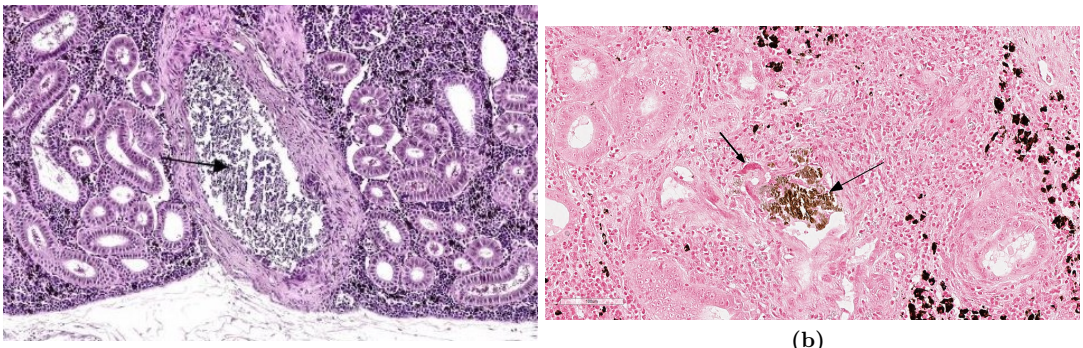
After transfer to sea the prevalence of nephrocalcinosis was reduced in fish groups with mild degrees of nephrocalcinosis (**Paper I**, Fig.10). The same has been observed by Nilsen et al. (2020); Lazado et al. (2022) and Fivelstad et al. (2003a). This might be due to a decrease in urine pH after transfer to sea, since Shehadeh and Gordon (1969) showed that urine pH decreases with increasing water salinity in rainbow trout. If the same is true for Atlantic salmon, the decreased urine pH may facilitate a dissolution of the mineral deposits with subsequent excretion of excess divalent ions. This should be further explored.

Another important aspect is that fish are capable of growing nephrons de novo throughout their life and that neo-nephrogenesis can be significantly increased if the kidney is acutely damaged (Davidson, 2014; Reimschuessel et al., 1990; Watanabe et al., 2009). It is possible that fish with mild nephrocalcinosis may regenerate sufficient amount of kidney tissue to obtain adequate kidney function after transfer to sea due to; both a stop in further deposition of calcium minerals with dissolution and excretion of excess ions, and a regeneration of damaged kidney tissue (Noble et al., 2018).

Mineral composition of deposits characteristic for nephrocalcinosis

Smart et al. (1979) used both chemical analysis of kidney tissue and different histological staining methods to demonstrate that nephrocalcinosis in rainbow trout consisted of calcium. Calcium usually stains purple-blue with haematoxylin and eosin (H&E) staining (Fig.11a), while the classic staining method of von Kossa, uses silver nitrate to demonstrate the presence of calcium in the tissue section giving a black color to calcium deposits (Suvarna et al., 2019), Fig.11b). One of the advantages of Von kossa staining is that it will only show phosphate and carbonate radicals, giving good results with both large and small deposits of calcium (Suvarna et al., 2019). On the other hand the method is not specific, as melanin will also reduce silver to give a black deposit (Suvarna et al., 2019). Histopathology will also show tissue reactions and damages caused by the deposits characteristic for nephrocalcinosis (**Paper I, II, III**).

Attenuated total reflection (ATR) Fourier transform infrared spectroscopy (FTIR) was used to examine deposits from Atlantic salmon diagnosed with nephrocalcinosis (**Paper I**). The spectra obtained were interpreted using an in-house library constructed for human kidney stones, which was modified for salmon nephrocalcinosis analysis. The results revealed the presence of five different minerals: amorphous carbonate apatite (amCAP), struvite, brushite, whitlockite and newberyite.



(a) (b)

Figure 11: Presence of deposits characteristic for nephrocalcinosis in kidney tissue from Atlantic salmon stained with H&E (a) and von Kossa (b). Arrows denote calcium deposits. Photo credit: Pharmaq Analytiq AS.

amCAP was the most prevalent mineral and was found in all but one of the mineral samples (Tab.1). Struvite, brushite, whitlockite and newberyite were found in different combinations together with amCAP, where amCAP constituted between 68 and 98% of the sample. There were differences in mineral composition between different hatcheries, but only a week significant correlation between mineral composition and nephrocalcinosis severity (p=0.04, rho = 0.21). Hench, fish with more severe nephrocalcinosis had a tendency of having a higher variation in mineral composition than fish with only mild nephrocalcinosis.

Table 1: Mineral complexes found in Atlantic salmon reared in commercial hatcheries in Mid-Norway (n=69)

Mineral complex	**Chemical formula**	**Samples (n)**	**Proportion (%)**
Amorphous carbonate apatite	$Ca_{10}(PO_4)_6CO_3$	68	98.6
Struvite	$MgNH_4PO_4$ x 6 H_2O	6	8.7
Brushite	$CaHPO_4xH_2O$	7	10.2
Whitlockite	$MgCa_8(PO_4)_6$	10	14.5
Newberyite	$MgHPO_4$ x 3 H_2O	8	11.6

In humans, both high and low pH can result in precipitation of minerals in the urine, depending on the chemical composition of the deposits (Han et al., 2019). It is not unlikely that the urine pH is relevant for nephrocalcinosis in fish as well (Khan and Glenton, 2008; Lewisch et al., 2013). Data on pH levels in urine in Atlantic salmon are scars, with only one publication of pH 7.5 in seawater acclimatised salmon (800 g) (Roy and Lall, 2004). In fresh water acclimatised rainbow trout (200 g) urine pH in unfed fish were 7.38 and increased after feeding to 7.78 (Bucking et al., 2010). Patel et al. (2006), Wood et al. (1999) and Perry et al. (1988) found comparable urine pH levels for fed and unfed rainbow trout, while Shehadeh and Gordon (1969) reported urine pH of 7.2 and 6.3 in fresh water and sea water acclimatised rainbow trout respectively. It is possible that urine pH in Atlantic salmon is in the same range as in rainbow trout and this should be examined in future studies on nephrocalcinosis in Atlantic salmon.

Calcium phosphate and struvite calculi appears to be overrepresented in fish (Gillespie and

Evans, 1979; Smart et al., 1979; Bjerknes et al., 1994; Béland et al., 2020; Applegate et al., 2016). According to Prof. Moe (pers. comm.), amCAP indicates rapid precipitation and the main risk factor for calcium phosphate kidneys stones in humans are high urine pH (Coe, 2005). The formation of carbonate apatite (CAP) begins at pH $\geq$ 6.8, with an increasing ability to aggregate with increasing pH (Olszynski et al., 2015). Provided that amCAP and CAP have similar chemical properties, it is possible that the urine pH of salmon may predispose it for calcium phosphate precipitation in the kidney.

We also found other mineral complexes together with amCAP (**Paper I**, Tab.1), but they never constituted more than $\frac{1}{3}$ of the mineral deposit. Newberyite is thought to be a breakdown product of struvite, since it is a very rare constituent of urolithiasis (Uebelhart et al., 1984), while brushite will hydrolyse to hydroxiapatite in alkaline pH (Klee et al., 1991). In human medicine struvite is always associated with bacterial infections (Karki and Leslie, 2022) and kidney stones with more than 20% whitlockite also indicate infection (Bazin et al., 2022). We cannot exclude the possibility that fish with high proportion of whitlockite and/or struvite/newberyite may have had an infection. Pathogen screening of a random selection of samples from four different strains and six different hatcheries were performed (Tab.2). Seven of the samples were positive for infectious pancreatic necrosis virus, although with low amount of virus detected. Non of the other common fish pathogens were detected in the kidney tissue of the sampled fish (Tab.2).

Table 2: Pathogen screening of kidney tissue from four different strains of Atlantic salmon reared in commercial hatcheries in Mid-Norway (n=13). *Aeromonas salmonicida* subsp. (Aeromonas), *Renibacterium salmoninarum* (BKD), *Flavobacterium psycrophilum* (Flavo), Infectious pancreatic necrosis virus (IPN) *Pasteurella* sp. (Pasteurella), *Vibrio anguillarum* O1 (Vibiro O1), *Vibrio anguillarum* O2A (Vibrio O2A), *Yersinia ruckeri* (Yersinia). qPCR with ct-values shown. ND = Not detected.

Fish ID	1	2	3	4	5	6	7	8	9	10	11	12	13
Aeromonas	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
BKD	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Flavo	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
IPN	ND	ND	ND	ND	ND	34.0	34.2	31.1	32.9	33.3	32.1	ND	33.4
Pasteurella	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Vibrio O1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Vibiro O2A	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Yersinia	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Etiology of nephrocalcinosis in juvenile farmed Atlantic salmon

In human and veterinary medicine, the determination of kidney stone composition play an important role both in treatment and prevention (Tepeler and Turna, 2017; Kourambas et al., 2001; Koehler et al., 2009; Kravdal et al., 2015). With this in mind the mineral deposits characteristic for nephrocalcinosis in Atlantic salmon may point to the etiology of the disease.

Calcium phosphate stones consisting of more than 30% amCAP are uncommon both in humans (Pers. comm. Prof. M.K. Moe) and domestic animals (Osborne et al., 2009), but when ob-

served they are linked to hypercalcemia, hyperparathyroidism, hypervitaminosis D, dystrophic and ectopic mineralization of vital tissues, distal renal tubular acidosis or urinary tract infections (Daudon and Jungers, 2012; Osborne et al., 1995).

Hypercalcemia and osmoregulatory stress

The plasma concentration of calcium, but also magnesium and phosphate were significantly elevated in fish with nephrocalcinosis (**Paper I, II**). In addition, the proportion of fish with hypercalcemia increased with increasing severity of nephrocalcinosis. An increase in plasma calcium and magnesium levels were also observed prior to a significant increase in nephrocalcinosis prevalence in a case study (**Paper IV**, Fig.12, 13). The elevated levels of calcium and magnesium were detectable for two consecutive months followed by a reduction to homeostasis. It is noteworthy that the prevalence of fish with nephrocalcinosis increased significantly when the plasma calcium levels were reduced proposing that secretion over the kidney resulted in precipitation of calcium minerals.

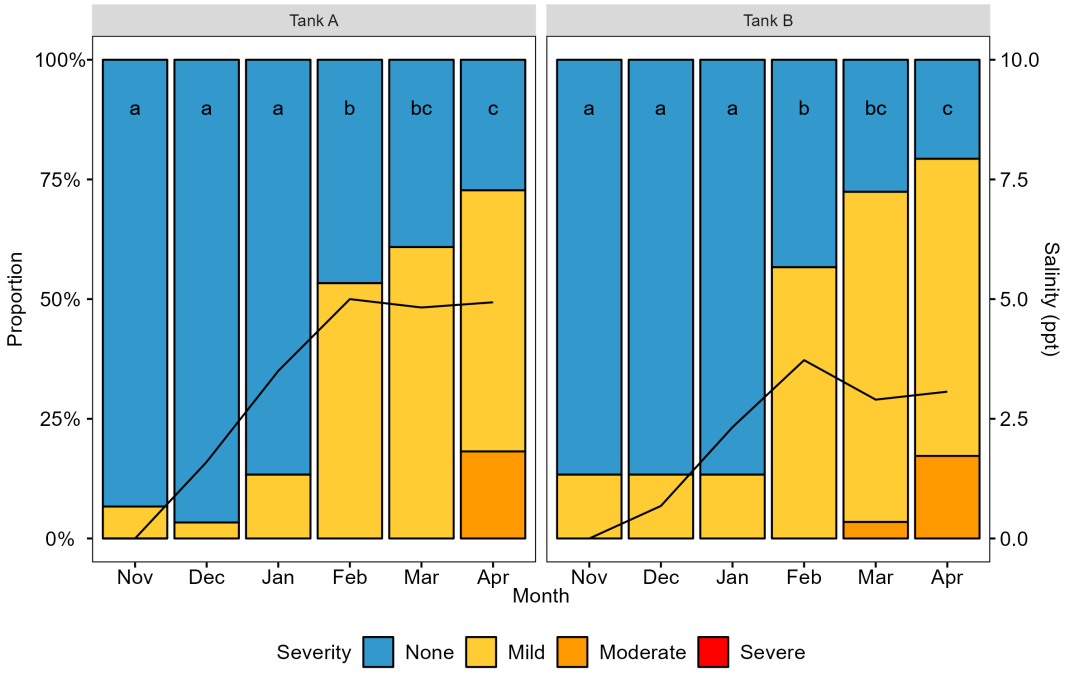


Figure 12: Prevalence and severity of nephrocalcinosis at different sampling points for tank A and B including salinity. Significant differences in nephrocalcinosis prevalence between sampling months is denoted with different letters ($p < 0.05$). Fish from tank A: Nov; n=15, Dec-Feb; n=30, Mar; n=23, Apr; n=22. Fish from tank B: Nov; n=15, Dec-Feb; n=30, Mar; n=29, Apr; n=29). Black line show salinity changes over time in tank A and B (ppt).

The predominant route of Ca^{2+} entry from the environment is across the gill epithelium (Lin

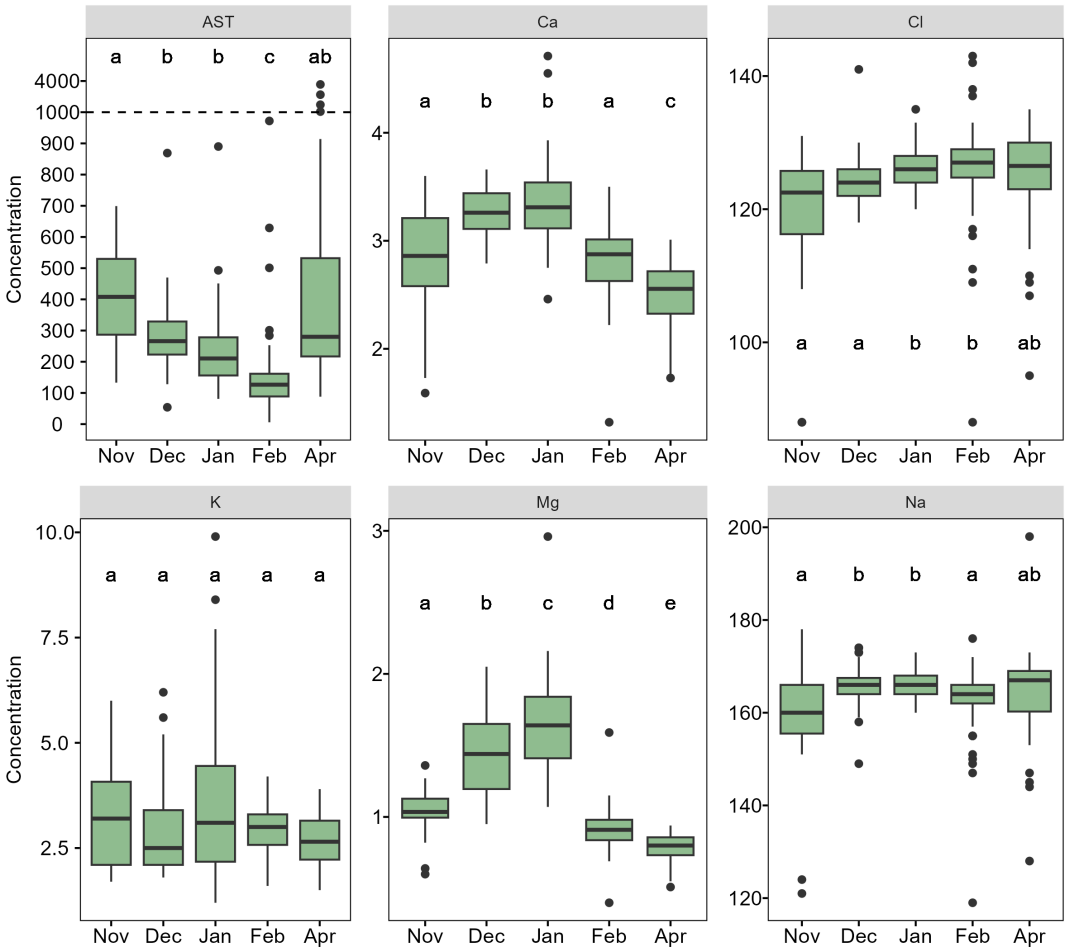


Figure 13: Blood chemistry parameters in Atlantic salmon at different time points during parr-smolt transformation: aspartate aminotransferase (AST), calcium (Ca), chloride (Cl), potassium (K), magnesium (Mg), sodium (Na). Significant differences between months are indicated with different letters ($p > 0.05$). For AST, the y-axis is drawn together for values above 1000 (marked with the horizontal dashed line). Fish: Nov; n=30, Dec; n=59, Jan-Feb; n=60, Apr; n=49.

et al., 2011; Flik and Perry, 1989) and the Ca^{2+} uptake function in fish has to be well regulated for maintaining the internal Ca^{2+} homeostasis during acclimation to fluctuating Ca^{2+} water levels (Evans et al., 2005). The increase in calcium concentration in December and January (Fig.13) was similar to what is seen after a 24-hour seawater challenge test (Nieves-Puigdoller et al., 2007) and after transfer to sea. According to Urke et al. (2014) homeostasis is usually achieved within two weeks after exposure to seawater, but in our case study (**Paper IV**) the fish experienced long-term (6-8 weeks) hypercalcemia causing chronic hyperosmotic stress. This could be a result of gradually increasing the salinity (Divino et al., 2016), since the calcium concentration in plasma was reduced to homeostasis when the salinity of the production water was kept stable (Fig.12). Mount et al. (1997); Goodfellow et al. (2000); Schwarz and Allen

(2014) have all suggested that salinity fluctuations may cause osmotic stress in fish.

Alternatively the link between increasing salinity and hypercalcemia could be due to premature introduction of brackish water to fish without sufficient hypoosmoregulatory capacity, also resulting in osmoregulatory stress (**Paper IV**). Changes in gill NKA α-1a isoform (Fig.14) indicated that the salinity was introduced to the fish before they had obtained sufficient hypoosmoregulatory capacity. The gene expression patterns in the kidney also indicating that the salinity increase was introduced while the kidneys were still adapted to the FW environment (Fig.15). It is possible that the duration (6-8 weeks) of osmoregulatory stress resulted in an over saturation of the urine when the fish finally began to osmoregulate properly which again may have facilitated the precipitation of calcium phosphate in the kidney.

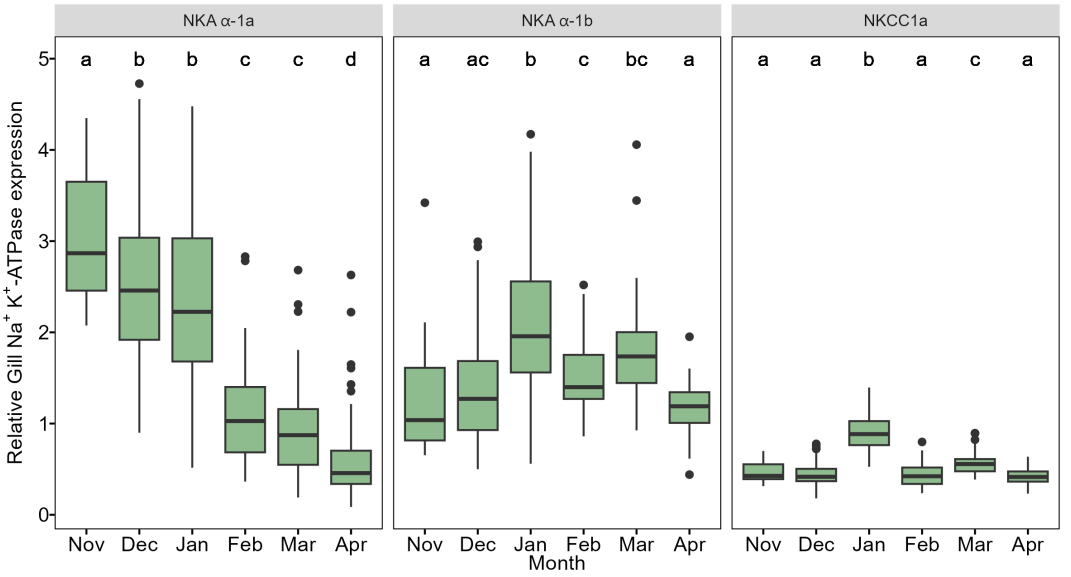


Figure 14: Box-and-whisker plots showing log2 qPCR expression ratios of genes related to fluid and electrolyte homeostasis in gill tissue over time. qPCR expression ratios are normalized to the geometric mean of expression ratios of elongation factor ef1α. Na^+,K^+-ATPase α-1a (α-1a), α-1b (α-1b) isoform and Na^+,K,$2Cl^-$ (NKCC1a) cotransporter.Fish: Nov; n=30, Dec-Feb; n=60, Mar; n=52 Apr; n=51 (**Paper IV**)

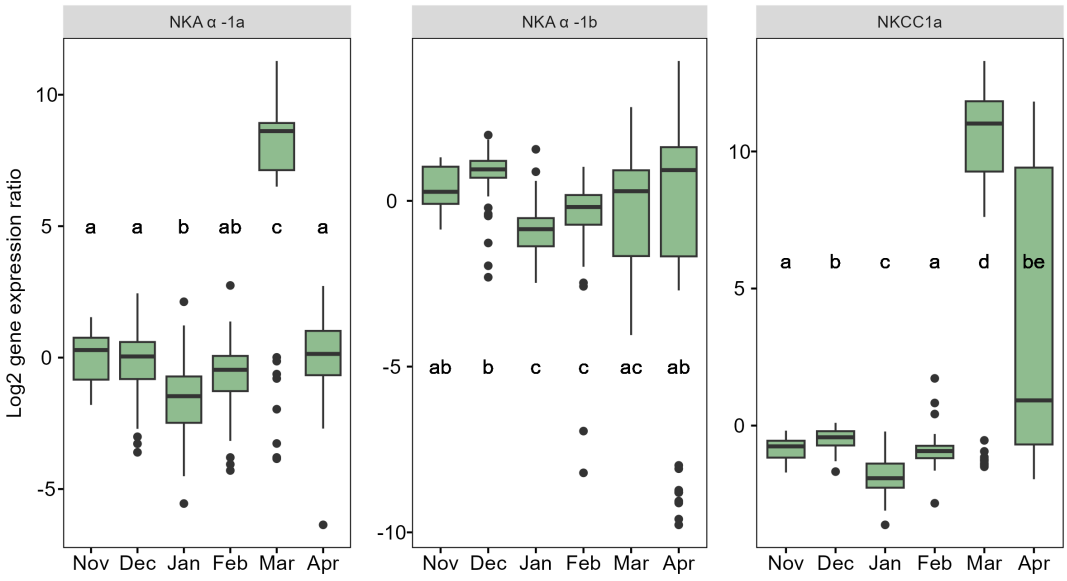


Figure 15: Box-and-whisker plots showing log2 qPCR expression ratios of genes related to fluid and electrolyte homeostasis in kidney tissue over time. qPCR expression ratios are normalized to the geometric mean of expression ratios of elongation factor ef1α. Na^+,K^+-ATPase α-1a (α-1a), α-1b (α-1b) isoform and Na^+,K,$2Cl^-$ (NKCC1a) cotransporter. Fish: Nov; n=22, Dec; n=58, Jan; n=54, Feb; n=44, Mar; n=42 Apr; n=40 (**Paper IV**)

A partial least squares (PLS) regression model including husbandry information, feed composition and water quality parameters collected from hatcheries of 12 fish groups (Fig.10 a) indicated that the supplementation of seawater in the production water and sulphate concentration in the tank water were the two parameters that explained the prevalence of nephrocalcinosis most (Fig. 16, **Paper IV**). The model explained 74 % of the variation in the response variable (nephrocalcinosis). The sulphate concentration of the tank water could be directly related to the salinity of the operational water since concentration of sulphate is normally low in fresh water (Kristensen et al., 2009). Taken together it would seem that introducing salinity to Atlantic salmon before smoltification increases the risk of developing nephrocalcinosis. The most reasonable explanation for this is that fish without sufficient hypoosmoregulatory capacity will experience osmoregulatory stress when exposed to brackish water concentrations.

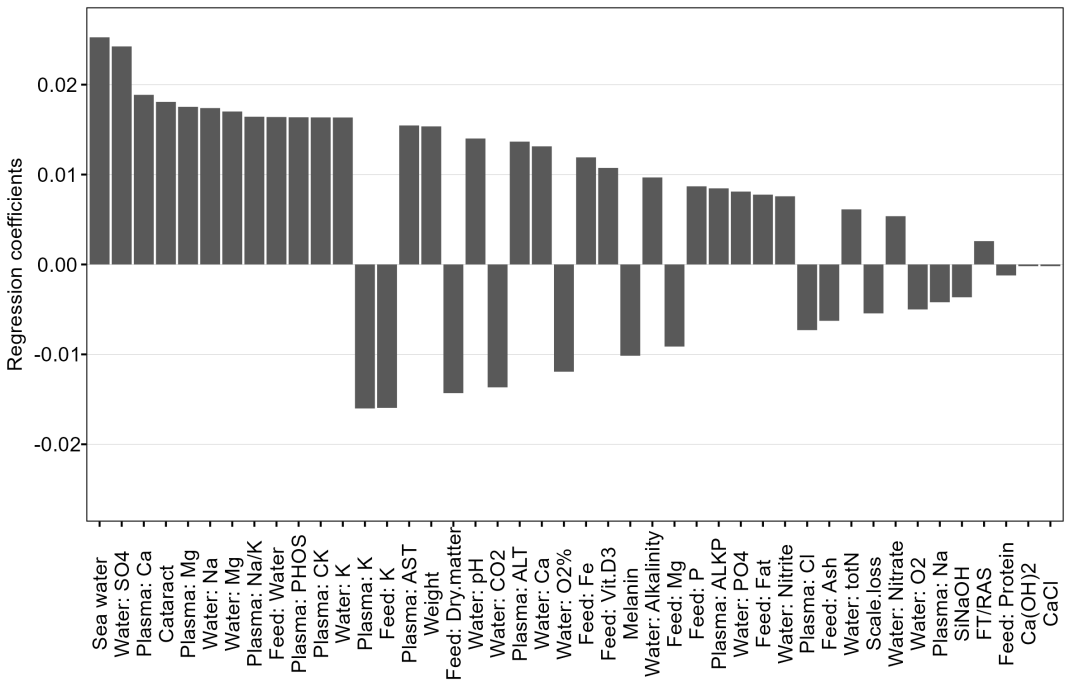


Figure 16: Regression coefficients from the PLS regression analysis of nephrocalcinosis with the covariates that were considered to have important contribution to the principal components in the full PCA. The covariates included rearing conditions, water quality measurements, feed composition and biological measurements and registrations (**Section: Supplementary material**). Root mean square error (RMSE): 0.34.

Osmoregulatory stress is the result of an increase or decrease in the concentration of solutes inside an organism or cell and poses a considerable threat to basic cellular functions required for survival (Evans, 2010). The effects of prolonged osmotic imbalance can be severe and result in damaging cellular macromolecules, disturbing cell volume, modifying stringent intracellular inorganic ion concentrations and altering intracellular macromolecular density (Kültz and Burg, 1998; Deane et al., 2002; Kültz, 2005).

Most of the osmoregulatory systems in fish rely upon predictable environmental salinities and are functionally restricted to either marine or freshwater habitats (Fiol and Kültz, 2007). Salmonids, on the other hand, can acclimatize to a range of environmental salinities through a series of complex adaptive processes that facilitate switching between hypotonic and hypertonic osmoregulatory systems (Kato et al., 2005; Tang and Lee, 2007) and these adaptive processes operate over a wide range of osmotic stress levels (Tipsmark and Madsen, 2001; Scott et al., 2004; Evans, 2002). These adaptive processes include mechanisms to rapidly modulate the activity of pre-existing osmotic effector molecules and minimize temporal delays between the onset of stress and the corresponding adaptive responses (Evans, 2010).

Wild Atlantic salmon will experience a relatively high, but short term osmotic stress during migration when the water salinity changes (Eddy, 1981; Hoar, 1988). The same is true for

farmed Atlantic salmon when transferred from the FW hatchery environment to the sea cages (Urke et al., 2014). On the contrary for farmed fish in hatcheries exposed to gradually increasing salinity over several months we would expect several connected episodes of hyperosmotic stress resulting in long term osmoregulatory stress. In wild Atlantic salmon there is no apparent requirement for a period of saltwater acclimation during migration (Moore et al., 1995), hence the protocol for smoltification or seawater adaption, with gradually increasing salinity, therefore appears to be poorly adapted to the salmon's natural smoltification behaviour. There is a knowledge gap on the effect of prolonged exposure to brackish water concentration on the health and welfare of farmed Atlantic salmon parr and smolts.

The use of SW supplementation to production water during the FW life stages of farmed Atlantic salmon is common in Norway (Toften, 2011). The salinity used ranges from 0.2 - 10 ppt for juvenile production and 0.2 - 33 ppt for smolt/post-smolt production respectively (Toften, 2011). Bœuf and Payan (2001) estimated that the metabolic costs of osmoregulation accounts for 5 to 50% of routine metabolic rate and these metabolic costs are likely to be highest in salmon reared in either FW or SW due to the large osmotic gradients between the internal and external environment (Hines et al., 2019). In theory the cost of osmoregulation should be reduced at intermediate salinities (Kristensen et al., 2012) and potentially increase growth rate and feed conversion, but may also affect tolerance to environmental stressors (Hines et al., 2019). The research findings on the potentially beneficial effects on fish health and performance of using SW supplementation to production water during the FW life stages of farmed Atlantic salmon are equivocal (Fivelstad et al., 2004; Handeland and Stefansson, 2002; Imsland et al., 2011; Duston, 1994; Saunders and Henderson, 1969; McCormick et al., 1989; Ytrestøyl et al., 2022).

Small changes in salinity (0 - 4 ppt) can cause significant changes in blood levels of divalent ions (Fig.13). This may be explained by the dramatic changes in the concentration of the major divalent ions Ca and Mg in low salinity ranges (Toften (2011)). To illustrate with an example, an increase in salinity from 0 ppt to 4 ppt gave a 6-fold increase in calcium concentration and a 47-fold increase in magnesium concentration, while an increase from 15 ppt to full strength SW will result in a 1.5-fold increase in calcium concentration and a 0.8-fold increase in magnesium concentration. This may imply that fluctuating salinity in low ranges will cause greater osmoregulatory stress than fluctuations in concentrations closer to full strength SW.

Marini and Kerstetter (1982) has previously suggested that electrolyte homeostasis, osmoregulation and mineral metabolism are closely related in salmonids. It has been demonstrated that embryonic exposure to road salt changes gene expression in Atlantic salmon including alterations suggesting interference with osmoregulation and mineral metabolism (Tollefsen et al., 2015). It is therefore possible that osmoregulatory stress in salmonids may alter mineral metabolism and thus result in precipitation of mineral salts in the kidney. Osmoregulatory stress may also contribute to increased adhesion for precipitation products through formation of crystal-binding receptors in the surface epithelium of the kidney tubules, as demonstrated in human cell lines exposed to stress (Vervaet et al., 2009). The mechanism for nephrocalcinosis in Atlantic salmon is not known, but in humans it is a multi-factorial process (Adams and Rowe, 1992). As previously described the urine pH of Atlantic salmon may facilitate precipitation of calcium phosphate complexes (**Section: Mineral composition of deposits characteristic**

for nephrocalcinosis), still there are commercial hatcheries that does not have high prevalence of nephrocalcinosis (**Section: Nephrocalcinosis - prevalence, severity and welfare implications**) pointing to the need for a trigger to develop nephrocalcinosis. This trigger may be osmoregulatory stress (**Paper IV**, Mulcahy et al. (1983)).

Exposure to high levels of environmental CO_2 concentrations

High levels of CO_2 in the ambient water was first suggested as a possible causative factor for nephrocalcinosis in 1979 (Smart et al., 1979). Since then several papers have both supported this notion (Fivelstad et al., 1999, 2018; Hosfeld et al., 2008; Damsgård et al., 2011; Foss et al., 2003; Fivelstad et al., 2003a) and failed to demonstrate this relationship (Fivelstad et al., 1998, 2007; Good et al., 2010, 2018; Mota et al., 2019; Martens et al., 2006; Graff et al., 2002). The possible link between high CO_2 levels in the ambient water and nephrocalcinosis development have been explained as an interference on normal kidney tubular function, resulting in alkaline tubular fluid and urine (Eddy et al., 1979). Precipitation of the mineral deposits characteristic for nephrocalcinosis in Atlantic salmon is favored by alkaline urine pH (**Section: Mineral composition of deposits characteristic for nephrocalcinosis**) which makes the link between high CO_2 and nephrocalcinosis plausible. Yet, none of the studies on the effect of high ambient CO_2 on health and performance in Atlantic salmon have measured urine pH. Without urine pH measurements the suggested link between high ambient CO_2 levels and alkaline urine pH is still an untested hypothesis.

It is possible that high ambient water levels of CO_2 may cause osmoregulatory stress. Mota et al. (2019) did find that fish exposed to high CO_2 had a thinner dermis and uneven epidermis, while Fivelstad et al. (2003b) observed gill damages as a result of CO_2 exposure in combination with low pH and aluminium in the water. Mota et al. (2020) also demonstrated that exposure to high CO_2 have an impact on gill tissue global gene expression. Changes or damages to epidermis and dermis, and gills may influence osmoregulation negatively (Bonga and Lock, 1992).

We did not find a correlation between environmental CO_2 levels and nephrocalcinosis prevalence or severity in our survey (**Paper IV**, Fig.16). It is also important to point out that all the hatcheries in the survey had CO_2-levels in the tank water below the recommended 15 mg/L which was regarded as low-medium CO_2-levels in Fivelstad et al. (1999, 2018); Hosfeld et al. (2008); Damsgård et al. (2011); Foss et al. (2003); Fivelstad et al. (2003a).

It is interesting to note that the studies performed on juvenile salmonids in FW where high CO_2 was suggested as causative factor, all have in common the detection of nephrocalcinosis in both experimental groups and in the control group (low CO_2) (Fivelstad et al., 1999, 2003a; Smart et al., 1979). It is also notable that the nephrocalcinosis was detected prior to the start of the experiment in three studies (Foss et al., 2003; Fivelstad et al., 2003a, 1999), indicating that high CO_2 was not in fact the trigger for developing nephrocalcinosis, but may have negatively contributed to the increase in prevalence of the condition in the experimental groups.

Possible nutritional factors

Knox et al. (1981) and Cowey et al. (1977) suggested that nephrocalcinosis in rainbow trout could be related to high levels of phosphorus and calcium and/or low levels of magnesium in feed. The same conclusion was drawn by Richardson et al. (1985) for Chinook salmon. The ratio tested by Cowey et al. (1977) and Knox et al. (1981) was approximately 400-700 while Richardson et al. (1985) was 66-72. Heinen et al. (1993) found differences in nephrocalcinosis incidence between different commercial rainbow trout feed, although not significant. The Ca:Mg-ratios in Heinen et al. (1993) were comparable to what is found in commercial feed for Atlantic salmon. It is also relevant to note that in the study, the nephrocalcinosis incidence were scored macroscopically and therefore might have been under- or overestimated. Commercial feed for juvenile Atlantic salmon usually contain a Ca:Mg-ratio of 5.5-8 (Tab.3), which is comparable to the groups that did not develop nephrocalcinosis in the studies by Cowey et al. (1977); Knox et al. (1981); Richardson et al. (1985); Heinen et al. (1993) which makes it less likely that the etiology of nephrocalcinosis is high Ca:Mg-ratios in feed in commercial salmon farming.

Table 3: Feed composition of commercial Atlantic salmon hatchery feed for the 12 fish groups shown in Fig.10 a. DW (dry weight).

Hatchery	1	2	3	4	5	6	7	8	9	10	11	12
Ash (%)	14	9.4	14	7.2	7.8	9.7	8.2	9.3	15	8.8	13	9.6
Calcium (g/kg DW)	14	16	16	12	11	17	13	17	20	18	16	15
Carbohydrate (%)	14	13	11	14	17	13	15	15	14	16	16	18
Chloride (g/kg DW)	51	9.7	37	7.8	5.5	14	8.8	14	51	8.5	25	7.2
Dry matter (%)	95	95	95	91	94	93	92	94	93	92	93	94
Fat (%)	23.7	22.3	28.6	21.7	22.3	22.9	21.7	19.9	18.7	21.3	21	21.1
Iron (mg/kg DW)	240	130	310	310	260	260	170	200	160	170	190	260
Potassium (g/kg DW)	11	13	8.8	10	11	11	11	12	11	11	9.8	12
Magnesium (g/kg DW)	2.3	2.7	2.0	2.3	2.4	2.6	2.4	3.0	2.8	2.4	2.1	2.4
Sodium (g/kg DW)	35	6.9	27	5.7	5.4	7.6	6.6	6.4	30	59	31	8.8
Phosphate (g/kg DW)	15	13	15	16	14	16	14	12	12	16	15	16
Protein (%)	44	50	41	48	47	48	47	50	46	46	43	45
Vitamin D3 (%)	6.54	7.68	12	8.36	5.93	6.93	5.32	5.2	5.45	6.16	7.25	6.12
Water (%)	5.4	5.1	5.4	9.3	5.5	7.1	7.7	6.3	6.6	8.1	6.9	6.1
Ca:Mg ratio	6:1	6:1	8:1	5:1	5:1	6:1	5:1	6:1	7:1	8:1	8:1	6:1

It is well known that hypervitaminosis D can result in irreversible calcification of soft tissues in humans and that hypercalcemia is an important marker for hypervitaminosis D (Pérez-Barrios et al., 2016). Tsertou et al. (2020) and Hilton and Ferguson (1982) did not find excess in vitamin D_3 to be related to the incidence of nephrocalcinosis in meagre (*Argyrosomus regius*) or rainbow trout and we did not find a link between vitamin D_3 levels in feed and nephrocalcinosis prevalence or severity (Fig.16, **Paper IV**). Further more, Atlantic salmon smolts showed no signs of hypervitaminosis D when fed large amounts of dietary vitamin D3 (Horvli et al., 1998), which contradicts hypervitaminosis D to be the most likely etiology of nephrocalcinosis in salmon.

Another nutritional factor that has been suggested to cause nephrocalcinosis is high levels of arsenic in feed (Cockell et al., 1991), however mild nephrocalcinosis was only observed in one of the experiments and not in the two others which may imply that the findings were random. It is also important to point out that the arsenic levels suggested to give nephrocalcinosis were 4 to 11 times higher than what is found in commercial salmon feed today.

High levels of dietary selenium (> 10 mg Se/kg) have also been proposed as a causative factor for nephrocalcinosis (Hilton and Hodson, 1983; Hicks et al., 1984), but may not be relevant. The reason for this is that commercial Atlantic salmon feed have changed the last decades with a reduction in fish meal content and concomitant increase in plant protein sources (Prabhu et al., 2020). This has reduced the selenium supply in the feed (Betancor et al., 2016; Fontagné-Dicharry et al., 2015; Sissener et al., 2013) and reduced the dietary availability (Prabhu et al., 2014; Silva et al., 2019). Commercial feed for Atlantic salmon today contains an average of 0.6 mg Se/kg feed (ranging from 0.3 to 1.1 mg Se/kg) (Sanden et al., 2016). It is therefore unlikely that high levels of selenium in feed is the causative factor for nephrocalcinosis in commercial Atlantic salmon farming.

Both Golomazou et al. (2006) and Mulcahy et al. (1983) concluded that it was unlikely that differences in nephrocalcinosis prevalence was related to dietary factors, supporting the findings in this thesis. We did not find a correlation between feed composition and nephrocalcinosis prevalence or severity (**Paper IV**), which makes feed a less likely causative factor for nephrocalcinosis.

CONCLUSION AND PERSPECTIVES

Nephrocalcinosis in farmed Atlantic salmon is a widespread production disorder that affects fish health and welfare negatively. It is therefore essential to find causative factors for nephrocalcinosis to be able to implement preventive measures. There are large variations in prevalence and severity of nephrocalcinosis in Norwegian hatcheries pointing to the environment rather than genetics as likely explanatory factor. In order to disclose possible environmental factors related to nephrocalcinosis, it was crucial to analyse the mineral deposits characteristic for nephrocalcinosis. The results showed that the mineral deposits mainly consist of calcium and phosphate ions, and precipitation of these minerals are favoured by alkaline urine pH. There is a lack of data on urine pH in Atlantic salmon and this should therefore be prioritized in future studies on nephrocalcinosis. If urine pH in Atlantic salmon are alkaline, it may be interesting to test if feed adaptions could be made to lower the urine pH and in turn prevent crystallization of calcium phosphate in the kidney as is done for domestic animals with kidney stones (Finke and Litzenberger, 1992). It is also possible that low urinary citrate (hypocitraturia) may be a risk factor. In human medicine, defects in the acid/base regulation has been seen to play an important role for the pathogenesis of nephrocalcinosis (Evenepoel et al., 2015). It has also been observed that metabolic acidosis reduces the concentration of citrate that is excreted in the renal tubules and that citrate works as a natural inhibitor of calcium phosphate precipitation. Lau (1989) therefore suggested that reduced urine citrate concentration may be linked to the development of nephrocalcinosis in humans. We do not know if hypocitraturia may be relevant for nephrocalcinosis development i farmed salmon, but it would be interesting to investigate a possible connection, since citrate supplementation of the feed can be a relatively simple measure to implement in commercial salmon farming.

The main risk factor uncovered in this thesis was omsoregulatory stress. Osmoregulatory stress may be induced by different environmental stressors, such as premature exposure to salinity, abrupt changes in water chemistry, and chronic exposure to suboptimal water quality. It is therefore important for salmon producers to monitor nephrocalcinosis development in their own facility in order to identify where in the production there is an increased risk for nephrocalcinosis occurrence and aggregation. Typical vulnerable phases in production are moving fish between different departments with subsequent changes in water quality, vaccination or other stressful handling procedures, changes in salinity and introduction of salt feed. Large variations in water quality should therefore be avoided, since abrupt changes in water ion concentration

causes osmoregulatory stress in fish. Transferring fish from FT to RAS and the other way around seems to be a risk factor which can be explained by the massive change in water chemistry between the two production forms.

There are several different strategies for inducing SW tolerance in farmed salmon, and a number of different smolt protocols in Norwegian hatcheries. Regardless of smolt protocol it is important to avoid mismatch in smoltification signals. If for instance salinity increase is used in combination with changes in photoperiod, the increase in salinity should be avoided before start of 24:0 light cycle. There is a delay in seawater adaption in the kidney compared to the gills, but both organs undergo a preadaption. If seawater is added when the fish is adapted to FW the gills and kidney will not handle the increase in ion concentration sufficiently. There is a risk for increased ion concentration internally in the fish due to a continued uptake of ions over the gills and reabsorption in the kidney leading to osmoregulatory stress. Future studies on nephrocalcinosis should include inducing osmoregulatory stress under controlled conditions.

Radiological detection of nephrocalcinosis provides the opportunity for non-invasive monitoring of development of nephrocalcinosis on an individual level. The method can be used to test different measures to investigate if nephrocalcinosis is reversible and to what extent. Radiology would therefore be suitable for controlled testing of measures to prevent or reverse nephrocalcinosis development.

The salmon farming industry in Norway rates nephrocalcinosis as one of the most important causes of reduced welfare in salmon, and there is great interest and impatience for obtaining effective measures. Controlled testing of various measures with documentation of effect or lack of effect can be a natural way forward in dealing with this challenge.

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SUPPLEMENTARY MATERIAL

Environmental measurements for PLS regression

The average value for each parameter for each fish group was used in the PLS regression model.

Table 4: Feed composition from fish group (fish gr.) 1-12; Ash (%), Fat(%), Protein(%), Dry matter (D. mat., %) Water content (Water, %), Vitamin D3 (D3, IU/g dry weight), Calcium (Ca, g/kg dry weight), Chloride (Cl, g/kg dry weight), Iron (Fe, mg/kg dry weight), Potassium (K, g/kg dry weight), Magnesium (Mg, g/kg dry weight), Sodium (Na, g/kg dry weight), Phosphorus (P, g/kg dry weight).

Fish gr.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Parameter												
Ash	14±1.1	9.4±0.8	14±1.1	7.2±0.6	7.8±0.6	9.7±0.8	8.2±0.7	9.3±0.8	15±1.2	8.8±0.7	13±1.0	9.6±0.8
Ca	14±1.1	16±1.2	16±1.2	12±0.9	11±0.8	17±1.3	13±1.0	17±1.3	20±1.5	18±1.4	16±1.2	15±1.1
Carb.	14±1.5	13±1.4	11±1.2	14±1.5	17±1.9	13±1.4	15±1.7	15±1.7	14±1.5	16±1.8	16±1.8	18±2.0
Cl	51±5.1	9.7±1.0	37±3.7	7.8±0.8	5.5±0.6	14±1.4	8.8±0.9	14±1.4	51±5.1	8.5±0.9	25±2.5	7.2±0.7
D. mat.	95±3.8	95±3.8	95±3.8	91±3.6	94±3.8	93±3.7	92±3.7	94±3.8	93±3.7	92±3.7	93±3.7	94±3.8
Fat	23.7±1.7	22.3±1.6	28.6±2.0	21.7±1.5	22.3±1.6	22.9±1.6	21.7±1.5	19.9±1.4	18.7±1.3	21.3±1.5	21±1.5	21.1±1.5
Fe	240±24	130±13	310±31	310±31	260±26	260±26	170±17	200±20	160±16	170±17	190±19	260±26
K	11±1.1	13±1.3	8.8±0.9	10±1.0	11±1.1	11±1.1	11pm1.1	12±1.2	11±1.1	11±1.1	9.8±1.0	12±1.2
Mg	2.3±0.3	2.7±0.3	2.0±0.3	2.3±0.3	2.4±0.3	2.6±0.3	2.4±0.3	3.0±0.4	2.8±0.4	2.4±0.3	2.1±0.3	2.4±0.3
Na	35±3.5	6.9±0.7	27±2.7	5.7±0.6	5.4±0.5	7.6±0.8	6.6±0.7	6.4±0.6	30±3.0	59±5.9	31±3.1	8.8±0.9
P	15±1.9	13±1.6	15±1.9	16±2.0	14±1.8	16±2.0	14±1.8	12±1.5	12±1.5	16±2.0	15±1.9	16±2.0
Protein	44±4.4	50±5.0	41±4.1	48±4.8	47±4.7	48±4.8	47±4.7	50±5.0	46±4.6	46±4.6	43±4.3	45±4.5
D3	6.54±1.3	7.68±1.5	12±2.4	8.36±1.7	5.93±1.2	6.93±1.4	5.32±1.1	5.2±1.0	5.45±1.1	6.16±1.2	7.25±1.5	6.12±1.2
Water	5.4±1	5.1±1	5.4±1	9.3±1	5.5±1	7.1±1	7.7±1	6.3±1	6.6±1	8.1±1	6.9±1	6.1±1

Table 5: Rearing conditions for fish group 1-12; stocking density (density), production technology (FT/RAS), sea water, SiNaOH $Ca(OH)_2$ and CaCl.

Fish group	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Density (Kg/m^3)	66.9	75.0	51.0	90.0	66.8	56.0	60.2	54.8	56.3	80.0	40.0	81.5
FT/RAS	FT	FT	RAS	FT	FT	FT	FT	FT	FT	FT	RAS	RAS
Sea water	no	no	yes	yes	no	yes	yes	no	no	no	yes	no
SiNaOH	no	yes	no	no	no	yes	no	yes	no	yes	no	no
$Ca(OH)_2$	no	no	yes	no	yes	no	no	no	no	no	no	yes
CaCl	no	no	yes	no	no	no	no	no	no	no	no	yes

Table 6: Water quality measurements from fish group (F.gr.) 1-12 (n=3 in each group); Alkalinity (Alk, ppm $CaCO_3$), Ammonium (Amm, mg/L) (Oxygen saturation (O_2, mg/L), Iron (Fe, mg/L), Potassium (K, mg/L), Sodium (Na, mg/L), Magnesium (Mg, mg/L), Phosphate (PO_4, mg/L), Sulphate (SO_4, mg/L), temperature (Temp, °C), pH, Nitrite (NH_3, mg/L), Nitrate (NH_4, mg/L), Total Nitrogen (totN, mg/L), carbon dioxide (CO_2) and turbidity (turb, NTU). Missing values are replaced by predicted values using the NIPALS algorithm and marked with *.

Fish gr.	1	2	3	4	5	6	7	8	9	10	11	12
Parameter												
Alk	3.2*	3.7±0.2	39.3±3.3	36.7±5.2	1.7±0.5	2.0±0.4	12.6±3.5	4.4±0.3	15.1±3.5	6.1±0.9	62.3±1.7	43.3±0.5
Amm	0.9±0.05	2.0±0.00	1.3±0.10	0.7±0.0	0.4±0.05	0.2±0.04	0.2±0.2	0.5±0.05	0.3±0.09	0.6±0.0	0.7*	0.3±0.0
Ca	5.7±0.9	19.0±2.2	125.0±9.4	157.0±48.9	16.7±3.1	30.9±13.5	76.0±1.4	15.7±3.4	36.0±3.6	18.0±0.8	208.0±5.7	160.0±16.9
CO_2	8.7*	6.7±0.5	6.0±0.8	4.7±0.5	10.7±1.3	2.0±0.0	2.0±0	3.3±0.5	3.0±0.8	2.3±0.5	1.7±0.9	8.3±0.9
Fe	0.1±0	0.1±0.01	0.1±0.01	0.0±0	0.1±0	0.1±0.03	0.0±0	0.1±0	0.0±0.01	0.0±0.0	0.0±0.0	0.1±0.0
K	18.7±5.4	6.3±1.7	11.7±0.9	240.0±0.0	6.7±1.3	6.0±0.8	39.7±2.1	8.7±2.5	7.0±3.6	7.3±4.0	13.3±4.0	11.3±2.6
Mg	19.2±0.9	21.3±0.8	48.3±2.2	692.3±32.3	7.9±0.6	29.0±3.7	103.0±2.2	9.4±0.7	5.1±0.3	3.9±1.6	2.3±1.9	4.1±1.3
Na	11.0±5.0	18.0±0	689.3*	5966.7±235.7	14.7±2.5	146.7±28.6	923.3±55.6	17.3±1.3	6.3±1.3	13.3±0.5	88.7±1.7	376.7±9.4
NH_4	0.2±0	0.2±0	39.9±1.9	0.2±0	0.5±0.1	0.4±0.2	0.3±0.1	0.5±0.2	1.5±0.1	0.6±0.1	42.1±1.9	21.7±0.1
NH_3	0.0±0	0.0±0	0.3±0.01	0.0±0	0.0±0	0.0±0.0	0.0±0	0.0±0	0.0±0.0	0.0±0.0	0.7±0.0	0.0±0.0
O_2	9.9±0.1	12.9±0.2	10.7±0.4	10.7±0.2	10.4±0.1	10.9±1.4	11.8±0.2	12.1±0.1	13.7±0.7	10.7±0.1	9.6±0.2	9.9±0.1
O_2 (%)	81.6±0.9	98.7±1.3	90.4±3.2	82.6±1.9	85.0±0.6	81.3±0.3	86.9±0.6	89.5±0.4	106.4±3.7	84.7±0.5	95.2±0.8	97.2±1.0
pH	6.0±0.01	6.3±0.04	7.2±0.2	7.0±0.03	5.9±0.1	6.3±0.16	7.2±0.1	6.4±0.16	6.8±0.0	6.7±0.07	7.3±0.0	7.0±0.02
PO_4	0.2±0.03	0.2±0.01	5.9±0.29	0.1±0.01	0.0±0.01	0.6±0.34	0.0±0	0.0±0.01	0.0±0.01	0.2±0.1	3.2±0.1	1.2±0.0
SO_4[a]	2.0	2.0	43.0±0.8	70.0	2.0	36.3±3.9	70.0	2.0	2.0	2.0	4.7±0.5	6.7±1.7
Temp	7.3±0.0	3.6±0.1	7.3±0.1	4.7±0.1	6.3±0.0	3.6±0.2	2.8±0.1	2.6±0.3	4.3±0.1	5.3±0.2	15.1±0.3	14.6±0.1
totN	6.4±4.9	1.5±0.3	46.2±3.7	0.5±0	0.5±0.0	0.5±0.0	5.0±3.6	0.5±0	3.0±0.8	0.5±0.0	52.0±2.5	20.0±0.0
Turb	0.6±0.1	3.2±0.3	1.7±0.2	0.4±0.0	0.6±0.0	0.4±0.2	0.2±0.1	1.0±0.1	1.4±0.4	2.6±0.2	1.6±0.2	0.6±0.1

[a] Detection range 2-70 mg/L.

Biological measurements for PLS regression

The average value for each parameter for each fish group was used in the PLS regression model.

Table 7: Plasma measurements from fish group 1-12 (n=30 in each group); alkaline phosphatase (ALKP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) calcium (Ca), creatinine kinase (CK), chloride (Cl), potassium (K), magnesium (Mg), sodium (Na), Na/K ratio, inorganic phosphate (PHOS) (PO_4).

Fish gr.	1	2	3	4	5	6	7	8	9	10	11	12
ALKP	205.9±66.2	520.3±205.7	533.5±150.1	510.0±253.4	422.9±90.7	466.7±210.3	334.9±118.3	222.6±159.3	357.1±120.5	390.8±143.6	240.6±74.9	194.3±78.4
ALT	19.1±8.3	19.6±6.7	29.9±11.1	21.4±9.0	17.1±6.7	20.8±10.2	21.2±11.1	16.5±9.2	15.0±5.7	25.3±9.7	23.9±17.9	15.2±3.3
AST	59.1±28.5	203.1±107.5	1039.7±390.3	523.6±185.8	449.4±174.9	525.9±252.2	378.4±159.8	109.4±86.6	210.2±126.4	197.0±114.5	569.8±348.4	411.1±178.6
Ca	2.5±0.1	2.5±0.2	4.1±0.4	3.7±0.6	2.6±1.6	3.9±0.6	3.1±0.2	2.0±0.7	2.8±0.4	2.9±0.4	3.2±0.4	2.9±0.2
CK	665.7±744	1243.4±789	5867.6±2603	5350.4±2697	5919.6±2716	6737.2±3500	4739.0±3115	1318.3±1102	2945.8±2065	3368.6±3224	4713.7±2773	3124.6±2567
Cl	124.0±3.7	126.3±4.4	100.1±13.1	120.9±7.6	120.4±7.8	130.1±3.8	130.8±5.1	132.6±6.6	133.8±2.0	131.6±8.0	122.0±14.4	124.0±2.2
K	3.4±0.8	3.0±0.9	2.1±0.5	2.0±0.3	3.0±0.7	2.0±0.2	2.6±1.1	3.5±1.1	3.8±0.8	2.1±0.6	3.5±1.3	3.3±1.1
Mg	1.0±0.07	0.9±0.1	1.0±0.2	3.7±1.7	0.8±0.1	1.7±0.2	1.1±0.1	0.9±0.2	1.1±0.1	1.1±0.2	1.3±0.3	1.2
Na	155.9±4.2	160.0±3.6	141.3±13.0	155.3±7.6	153.0±8.0	163.3±3.3	164.3±5.1	162.5±7.1	164.2±1.8	163.1±8.1	156.9±11.3	156.6±2.4
Na/K	49.6	57.9	68.8	77.4	54.9	80.8	75.2	50.9	44.9	81.3	49.8	52.8
PHOS	3.2±0.6	4.2±0.6	5.4±0.7	7.2±1.0	4.7±0.5	6.3±1.1	5.5±1.0	4.1±1.1	3.8±0.7	5.8±1.2	8.4±1.4	5.9±0.9

Table 8: Biological measurements from fish group 1-12 (n=30 in each group); weight (g), length (cm), hearth deformities (yes(1)/no(0)), HSS (yes(1)/no(0)), light liver (yes(1)/no(0)), swollen spleen (yes(1)/no(0)), internal adhesions* (score 0-3), cataract (yes(1)/no(0)), fin injuries* (score 0-9), gill deformities* (score 0-3), jaw/snout wounds (yes(1)/no(0)), melanin* (score 0-9), scale loss* (acore 0-3), smolt characteristics (silvering, parr marks, dark tale fin edge, 1-4), gill ATPase ratio and strain (1-7).

Fish group	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Length (cm)	23.0±1.3	26.9±1.4	27.0±1.8	31.7±2.5	22.4±1.3	24.8±2.0	23.8±1.5	21.8±1.9	22.7±1.3	20.0±1.2	23.0±1.4	22.6±1.5
Weight (g)	133.6±25.6	211.1±32.3	222.1±51.5	384.7±85.8	119.7±20.6	108.2±19.8	148.0±27.6	125.9±35.7	125.2±21.0	97.0±17.7	138.4±21.6	111.7±22.0
Hearth deform.	0.0±0.2	0.0±0.2	0	0	0	0	0	0.1±0.3	0	0	0.0±0.2	0.1±0.3
HSS	0.1±0.3	0	0	0.0±0.2	0.0±0.2	0	0	0.1±0.3	0	0.0±0.2	0	0
Light liver	0.2±0.4	0.5±0.5	0.0±0	0.5±0.7	0.0±0.2	0.0±0	0.0±0.2	0.3±0.5	0.2±0.3	0.1±0.3	0.1±0.3	0
Swollen spleen	0	0	0	0	0	0.2±0.4	0.0±0.2	0	0	0	0	0
Adhesions	2.3±0.8	2.7±0.9	1.4±0.7	1.5±0.9	2.6±0.5	0.9±0.5	1.8±0.8	2.9±0.8	2.2±0.7	3.4±0.7	3.6±1.1	2.3±0.8
Cataract	0	0.4±0.5	0	0.8±0.4	0	0.3±0.5	0.8±0.4	0.3±0.5	0	0	0.0±0.2	0
Fin injuries	0.5±0.5	4.8±2.0	1.6±0.9	3.8±01.4	2.7±1.1	2.6±2.0	4.2±1.2	4.4±2.1	3.1±1.6	2.2±1.6	3.2±1.4	4.0±1.9
Gill deformities	0.2±0.5	0.2±0.4	0.1±0.3	0.1±0.4	0.1±0.4	0.1±0.4	0.1±0.3	0	0.8±0.9	0.1±0.3	0.4±0.6	0
Snout wounds	0	0	0	0	0	0	0	0	0	0.0±0.2	0	0
Melanin	2.7±1.0	1.9±0.6	1.2±0.5	2.9±1.4	2.7±0.7	1.3±0.8	1.9±1.0	1.9±0.3	2.5±1.0	1.7±0.5	1.4±0.7	3.0±0.8
Scale loss	0.1±0.3	0.7±0.8	0.1±0.3	0.1±0.4	0	0.0±0.2	0	0	0	0	0.1±0.3	0
Smolt state	3.9±0.2	3.5±0	4.0±0	4.0±0	3.7±0.2	3.8±0.3	3.8±0.3	3.7±0.4	3.5±0.2	2.7±0.5	4.0±0	3.8±0.3
ATPase ratio	1.1	1.3	2.4	0.4	1.4	0.7	0.9	0.9	0.4	1.7	0.8	1.9
Strain	1	3	4	5	1	5	7	3	6	7	1	7

*Operational well fare indicators scored according to FISHWELL (Noble et al., 2018).

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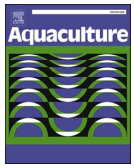

Physiological changes observed in farmed Atlantic salmon (*Salmo salar* L.) with nephrocalcinosis

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ABSTRACT

There is a growing concern for fish health and welfare in the salmon industry in Norway. Nephrocalcinosis, described as mineral deposits within the kidney, is increasingly observed. However, little is known about its frequency and severity in Norway. In this study 810 Atlantic salmon were sampled from 14 different fish groups in nurseries in Mid-Norway and receiving sea farm. Kidneys were examined for nephrocalcinosis by histopathological methods and all fish groups were diagnosed with nephrocalcinosis. The prevalence and severity of the disorder varied extensively between facilities. Most of the fish (68%) had mild forms of nephrocalcinosis, exhibiting at most, negligible tissue damage while fish affected by severe forms of nephrocalcinosis had an almost complete loss of kidney structure. Regardless of the severity of nephrocalcinosis, mineral deposits were mainly found in the form of amorphous cabonate apatite (amCAP), a calcium-dominated mineral. Accordingly, a majority of fish affected by nephrocalcinosis were diagnosed with hypercalcemia. Fish affected by moderate and severe forms of nephrocalcinosis also exhibited high levels of plasma magnesium, glucose, and aspartate aminotransferase (AST). These imbalances in plasma chemistry are likely to be an indication of disturbed osmoregulation and increased stress levels. The results of this study therefore suggest that nephrocalcinosis is a common and serious welfare challenge in Atlantic salmon that needs better monitoring.

1. Introduction

The salmon industry is one of the most important industries in rural Norway (Olaussen, 2018) employing over 8000 people and contributing to a yearly landing value of 6,8 billion EUR (NOK 70 billion) (Directorate of Fisheries, 2019). The industry is known for its innovation and use of new technologies, but welfare of farmed salmon is becoming a growing concern (Sommerset et al., 2020). Nephrocalcinosis is one of the challenges in Atlantic salmon and was among the major diseases listed by fish health professionals in the Norwegian Fish Health Report from 2019 (Sommerset et al., 2020). Although the prevalence of nephrocalcinosis among wild fish is unknown it is likely that the occurrence at production sites is related to the intensive conditions in aquaculture (Applegate et al., 2016; Béland et al., 2020; Bjerknes et al., 1994; Cavrois-Rogacki et al., 2021; Gillespie and Evans, 1979; Klosterhoff et al., 2015; Lewisch et al., 2013; Smart et al., 1979).

To date, there is no systematic registration of nephrocalcinosis in salmon aquaculture, however there is a growing effort to monitor the disease. A scoring form to visually document nephrocalcinosis is currently being validated by Nofima (Noble et al., 2018) and the condition has been more frequently reported by nurseries, fish farms and fish health personnel over the past few years, but without clear indication of severity and etiology (Sommerset et al., 2020). The condition is believed to arise during production on land (nurseries), with a progressive aggravation until transfer to sea (pers. comm. P.A. Sæther). It is generally accepted that nephrocalcinosis is related to increased mortality in the first weeks after sea-transfer and secondary infections with bacteria and fungi are not uncommon.

Nephrocalcinosis is described as deposits of mineral salts within kidney tubules and collecting ducts, which can be visually identified

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(Bruno, 1996). However, macroscopic assessment is not a precise tool, since small deposits are rarely visible to the naked eye (Klykken et al., 2020). To date, histopathology is therefore regarded as the standard in assessing the severity and progress of this condition. Observed changes involve basophilic mineral deposits that may progress into dilation of tubules and collecting ducts, degeneration and necrosis of epithelium of the affected tubular structures, and urine-stagnation causing dilatation and fibrosis in up-stream structures. Upon breach of the tubular structures, fibrosis and inflammation can cause extensive tissue reactions and necrosis in surrounding interstitial tissue (Fivelstad et al., 2018). In addition to the excretory system, the kidney also consist of both hematopoietic, immunological and endocrine tissues, all of which can be damaged upon development of lesions into the interstitial compartment. The sequential progression of histopathological changes occurring during the development of nephrocalcinosis makes it ideal for a scoring system. However, to the authors knowledge, such a scoring system for nephrocalcinosis based on histopathology has not been published. The development of a standardized scoring-system would be crucial to ensure a systematic approach when comparing degrees of tissue lesions both within and between groups, as well as their development over time.

Few studies have been conducted regarding the composition of kidney mineral deposits in farmed Atlantic salmon. To date, there is no peer-reviewed study on the composition of mineral deposits in salmon, but a master thesis from 2019 reported that the kidney deposits consisted of calcium and phosphate minerals (amorphous carbonate apatite, amCAP), carbonate apatite (CAP) and magnesium ammonium phosphate (struvite) (Thomsen, 2019). A survey conducted by Marin Helse AS on post-smolt salmon also reported that kidney deposits consisted of complexes of amCAP, with possible traces of complexes of magnesium, calcium and phosphate (whitlockite) (Sæther, 2019).Among other marine species, a study has been performed on Cobia, (*Rachycentron canadum*), where the kidney stones consisted of pure calcium, oxalate and calcium phosphate (Klosterhoff et al., 2015). In rainbow trout (*Oncorhynchus mykiss*) most studies found that the deposits consisted of calcium, phosphorus, carbonate and magnesium (Bjerknes et al., 1994; Gillespie and Evans, 1979), while Fíkrĭ et al. (2000) found that they contained ammonium urate ($NH_4C_5H_3N_4O_3$) and calcium phosphate.

In humans and domestic (terrestrial) animals, the determination of kidney stone composition play an important role both in treatment and prevention (Koehler et al., 2009; Kourambas et al., 2001; Kravdal et al., 2015; Tepeler and Turna, 2017). It is therefore likely that the mineral composition of nephrocalcinosis in salmon could provide relevant information to prevent the condition.

Biochemical analysis of plasma is a widely used diagnostic tool in human and veterinarian medicine. There is an increasing interest in transferring this methodology to the fish farming industry (Fazio, 2019), but the lack of standardized reference values from healthy fish makes it challenging to detect abnormalities (Clauss et al., 2008; Wade et al., 2019). One study proposed normal ranges of plasma chemistry for adult Atlantic salmon (Sandnes et al., 1988), but reference intervals have yet not been established for parr and smolt. Studies based on controlled experiments have demonstrated changes of plasma biochemistry in response to a variety of stressors (Calabrese et al., 2017; Iversen and Eliassen, 2009; Iversen et al., 2009, 1998; Stiller et al., 2020; Veiseth et al., 2006), toxins (Berntssen et al., 2018, 2021; Nieves-Puigdoller et al., 2007) and subtoxic concentrations of water quality compounds (Knoph and Thorud, 1996). Atlantic salmon with coldwater vibrosis (Waagbo et al., 1988) and cardiomyopathy syndrome (CMS) (Yousaf and Powell, 2012) also exhibited changes in blood biochemistry compared to healthy fish. Establishing biochemistry as a non-lethal diagnostic tool for early detection of nephrocalcinosis would represent a valuable tool for revealing risk factors and ultimately preventing the condition.

The objective of this study was to determine the prevalence and severity of nephrocalcinosis in farmed Atlantic salmon in Mid-Norway. To do so, we (1) sampled an extensive number of fish from commercial production groups, (2) examined the kidney tissue with histopathologic methods, (3) determined the composition of mineral deposits found in the kidney and (4) compared the plasma chemistry of individuals affected by nephrocalcinosis with healthy salmon.

2. Materials and methods

2.1. Data sampling

A total of 420 farmed Atlantic salmon were sampled from 14 fish groups in twelve different nurseries, both flow through (FT) and recirculation aquaculture systems (RAS) in Mid-Norway from October 2019 to April 2021. In addition, 390 fish originating from these nurseries were sampled from the receiving sea farm. Group 14 was not sampled after transfer to sea due to capacity issues at the facility. No fish were exposed to experimental manipulation, and the sample material therefore represents fish under conventional farming conditions (see Table A1 and A2 in supplementary material). A total of 30 fish from each facility were randomly sampled among visually healthy (normal swimming behaviour, absence of external injuries/lesions, no sign of emaciation) individuals within 2 weeks before transfer to sea and 1 month after transfer to sea. All sampling were performed in the morning (before 11 am). The fish were not starved before sampling, and they were euthanized with an overdose of Benzoak VET (200–400 mg/l) followed by a sharp blow to the head according to Norwegian legislation (Akvakulturdriftsforskriften, 2008). A general health assessment and physical observations including an evaluation of morphological changes related to parr-smolt transformation was performed at each sampling (parr-smolt transformation; body silvering, darkening of fin margins, loss of parr marks).

2.2. Blood collection and determination of plasma chemistry

Blood samples were collected from 420 fish that were sampled in nurseries. Vacutainer tubes (Becton-Dickinson, Rutherford, NJ, USA) with lithium heparin as anticoagulant were used to collect blood from the caudal vein, immediately after euthanasia. After thorough mixing, the samples were centrifuged at 13500 rpm for 5 min (VWR Mikrostar 12, 12 × 1.5/2.0 ml) and the plasma was transferred to Eppendorf tubes and kept frozen (minimum −20 °C) until analysis.

The following parameters were measured at Aqua Kompetanse's laboratory using an automated dry chemistry analyser (CatalystOne, IDEXX Laboratories, Westbrook, ME, (Boes et al., 2018)): alkaline phosphatase (ALKP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CREA), calcium (Ca), sodium (Na), potassium (K), chloride (Cl), magnesium (Mg), inorganic phosphate (PHOS), glucose (GLU), lactate (LAC) and urea. Other parameters that were derived included the following: sodium-to-potassium ratio (Na/K) and Osmolality (mmol/kg) calculated as:

$$1.86(Na + K) + 1.5GLU + UREA + 14$$

according to Martín-Calderón et al. (2015). Each of the assays used a standard kit developed for the automated analyser (CatalystOne, IDEXX Laboratories, Westbrook, ME). The plasma occasionally showed activities for inorganic phosphate exceeding the instrument ranges, and sporadically for other parameters: in these cases a physiological saline solution was used to dilute the samples (1:4).

2.3. Mineral deposits

When visually identified, mineral deposits were carefully collected from fish ureters and stored in Eppendorf tubes and frozen until analysis (−20°C). The samples were washed in ethanol (EtOH) followed by centrifugation and acetone to remove lipids and water from the samples and air dried for 5 min prior to analysis. The mineral composition was

examined by attenuated total reflection (ATR) Fourier transform infrared spectroscopy (FTIR) in a Nicolet iS10, operated by the Omnic9 software. The spectra obtained were interpreted by OmnicSpecta using an in-house library constructed for human kidney stones, which has been modified for salmon nephrocalcinosis analysis.

2.4. Histopathology

Tissues from the mid-kidney were sampled from all individuals for histopathological analysis of nephrocalcinosis. The kidney tissues were fixed in 4% formaldehyde solution, embedded in paraffin wax and routinely processed (Suvarna et al., 2019). All sections were stained by haematoxylin and eosin and a selection of sections were stained with von Kossa stain (Rungby et al., 1993). The histopathological diagnosis of nephrocalcinosis was defined as the presence of amorphous (structureless), basophilic deposits in tubules, collecting ducts and excretory ducts. Histopathological sections were analysed, and presence of deposit and degree of tissue damage was evaluated and given a score based on the type and distribution of changes. The nephrocalcinosis score defines and categorises the presence of deposits, degree of tissue damage in structures with deposits, glomerular alterations and pathology in the interstitial tissue. Pathological changes were divided into 4 subcategories (Table 1). Each category was weighted by the effect the different pathological changes are believed to have on the development of the condition and the time it will take to heal.

The score of the first category was weighted with a factor of 1, while the score of category 2 was weighted with a factor of 2 because dilation of tubules and renal collection ducts causes gradual damage on the epithelium and subsequent chronic changes resulting in fibrosis of the basal membranes of the affected structures. Urine stagnation with dilation of the glomerular space is indicative of down-stream obstruction (Docherty et al., 2006) and develops into periglomerular fibrosis and subsequent thickening of Bowmans capsule. For this reason the score of category 3 was weighted with a factor of 3. The score of category 4 was weighted with a factor of 4 since presence of deposits in the interstitial tissue over time results in necrosis and loss of the affected structures. Deposits in the hematopoietic- and immune tissue of the interstitium leads to granulomatous inflammation surrounding the damaged structures (tubules, collecting ducts and excretory ducts). The inflamed interstitial tissue is gradually replaced by fibrosis.

Table 1
Histopathological nephrocalcinosis score. The scores are weighted based on the effect the various changes are believed to have on the development of the disease and the time it will take to heal the condition.

Severity	1	2	3
Category 1 Presence of deposits	Sparse amounts in collecting ducts and ureteres - close to absence in tubules and affects less than 10% of the excretory system	Moderate amounts in collecting ducts and ureteres - sparse amounts in tubules and affects between 10% and 50% of the excretory system	Extensive quantities in ureteres, collecting ducts and tubules and affecting more than 50% of the excretory system
Category 2 Epithelial degeneration and/or necrosis	Affects less than 10% of tubules and collecting ducts	Affects between 10% and 50% of tubules and collecting ducts	Affects more than 50% of tubules and collecting ducts
Category 3 Pathological changes in the glomeruli	Dilatation of the glomerular space (urine stagnation) and fibrosis / thickening of the parietal Bowman's capsule, changes in less than 10% of the glomeruli	Dilatation / thickening of the parietal Bowman's capsule, peri-glomerular fibrosis - changes in between 10% and 50% of glomeruli	Dilatation, thickening of the parietal Bowman's capsule, peri-glomerular fibrosis, changes in over 50% of glomeruli
Category 4 Pathological changes in the interstitial tissue	Affects less than 10% of interstitial tissue	Affects between 10% and 50% of interstitial tissue	Affects more than 50% of interstitial tissue.

Total nephrocalcinosis score is calculated as:

$$\sum_{n=1}^{4} C_n \times S_{C_n}$$

Where C is the category, S is the severity and n is category number.

In the nephrocalcinosis score, overall scores 1 to 10 were generally considered mild changes, scores 11 to 20 were considered moderate changes and scores greater than 20 were regarded as severe changes.

2.5. Statistical analysis

The reference interval for normal ranges of plasma chemistry parameters were obtained by including all fish without nephrocalcinosis ($n = 234$) and excluding fish with HSS (Haemorrhagic smolt syndrome, $n = 5$), to ensure that only healthy fish were included in the data set ($n = 229$). Extreme values (outliers) were identified using Horn's algorithm with Tukey's interquartile (IQ) fences. The criterion for rejection was values exceeding IQ fences according to Horn et al. (2001). A non-parametric statistical method with a 95% confidence interval of reference intervals was chosen, were the 2.5th and 97.5th fractiles serve as the lower and upper reference limits (Friedrichs et al., 2012).

All statistical analyses were performed using R software 4.0.5 (R Core Team, 2017). Normality was tested with the Shapiro-Wilks test and showed non-normal distribution for the majority of plasma chemistry variables and the non-parametric test Kruskal-Wallis was performed to explore significant differences. Wilcoxon rank sum test was used to compare healthy fish and fish with different severity of nephrocalcinosis. *P*-values were adjusted using Bonferroni correction. P-values ≤ 0.05 were stated as significant.

3. Results

Visual assessment showed that 89% of the fish from the nurseries had developed physical smolt characteristics as described in Langdon (1985). There were no diseases documented among the nurseries, with exception of HSS in 1.7% of the sampled fish (0.5% had both HSS and nephrocalcinosis). The mean weight of healthy fish was 150.4 ± 91.2g, while the mean weight of fish affected by nephrocalcinosis was 270.0 ± 204.4g ($p<0.05$). The gender distribution was 49% males and 51% females in both the healthy group and the group affected by nephrocalcinosis.

3.1. Histopathology

Mild changes in the kidney primarily consisted of amorphous mineral deposits in collecting ducts (Fig. 1b), and tubulus (Fig. 1c) with minor changes in the epithelium of the tubular structures. With increased severity, increased damage to the tubular wall was seen. From degeneration and necrosis of the epithelium (Fig. 1d) to complete loss of epithelium with fibrosis of the basal membrane, and dilatation (Fig. 1e), and further to complete loss of integrity of the wall, often accompanied by extensive tissue reactions in surrounding interstitial tissue (Fig. 1f). Associated glomerular changes involved dilatation of the glomerular space, fibrosis and thickening of the parietal layer of the Bowman's capsule, and varying degree of per-glomerular fibrosis and glomerulitis (Fig. 1g). These changes observed in the glomeruli are thought to be at least in part a result of urine stagnation (Docherty et al., 2006). In advanced cases, acute interstitial inflammation or chronic interstitial fibrosis is seen (Fig. 1h), often in association with misshaped and degenerated tubuli (Fig. 1h) and extensive dilatation of collecting ducts,

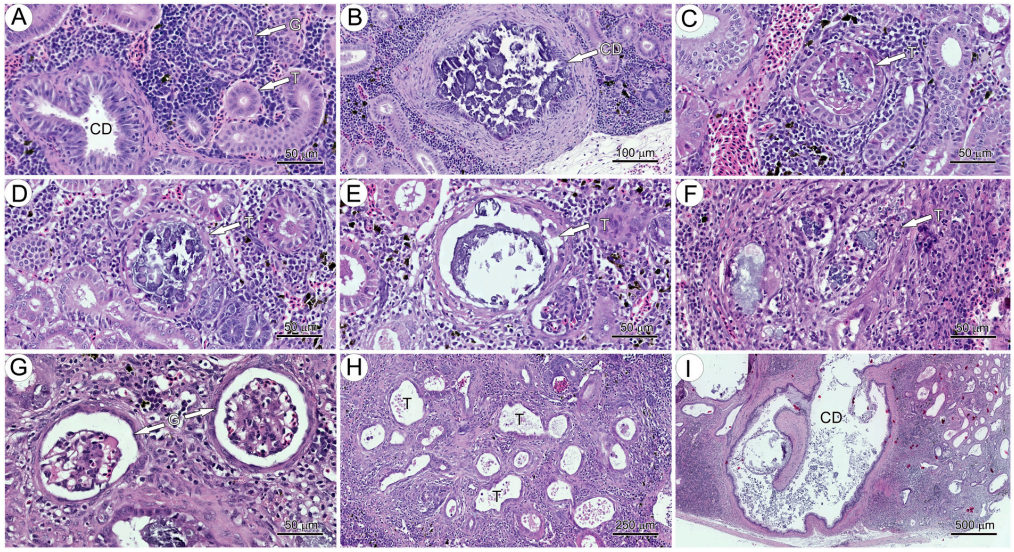

Fig. 1. Histopathological changes associated with nephrocalcinosis. A) Normal tissue showing a healthy glomerulus *(G)*, tubulus *(T)* and collecting duct *(CD)*. B) Early lesion, with basophilic, amorphous deposits in a collecting duct. Note that the epithelium of the duct is intact, and that surrounding tissue appears without remarks. C) Early lesion in a tubule, with slight occurrence of degeneration and necrosis of the tubular epithelium. Deposits together with necrotic epithelial cells are seen in the tubular lumen. D) Moderate lesion in a tubule, with marked degeneration and necrosis of the epithelium and rich amounts of deposits in the lumen. E) Extensive, chronic lesions in a tubule, with complete loss of epithelium, fibrosis of the basal membrane, and dilatation of the lumen containing deposits. F) Advanced, chronic lesion with complete destruction of the tubular structure including the basal membrane, allowing the deposits getting in contact with the interstitial tissue inducing massive inflammation. G) Chronic changes seen in glomeruli, with dilatation of the Bowman's space, fibrosis and thickening of the parietal layer of the Bowman capsule, peri-glomerular fibrosis and moderate glomerulitis. H) Chronic changes, with degeneration of epithelium and dilatation of tubules, surrounded by extensive fibrosis replacing normal interstitial tissue. I) Chronic changes, with extensive dilatation of a collecting duct, degeneration and necrosis of epithelium and presence of deposits together with necrotic cells in the lumen.

with degeneration and necrosis of associated epithelium (Fig. 1i). The changes observed in the interstitium appear when the mineral deposits formed within the excretory system breaks through the tubular wall and interacts with the immune tissue of the interstitium.

Black staining of deposits by von Kossa stain indicated high contents of calcium salts in the deposits associated with nephrocalcinosis (Fig. 2).

The most severe cases of nephrocalcinosis were easily visually identified, as extensive amounts of deposits cause extensive loss of kidney structure (Fig. 3).

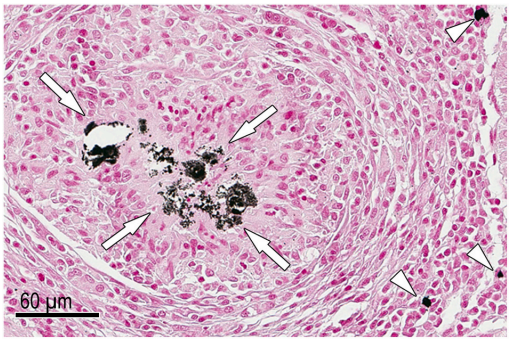

Fig. 2. von Kossa stain of kidney tissue with deposits and tissue reaction associated with nephrocalcinosis. Black staining (arrows) indicative of presence of calcium salt, surrounded by a granulomatous inflammatory process. Arrowheads points to melanin-containing cells; as for calcium salts, melanin reduces silver from the von Kossa stain into black deposits.

Fig. 3. Severe nephrocalcinosis in Atlantic salmon - extensive amounts of deposits in the kidney accompanied by swollen tissue and loss of normal structure.

3.2. Prevalence and severity of nephrocalcinosis in mid-Norwegian nurseries and sea farms

Nephrocalcinosis was observed in all the nurseries in this survey, even though the prevalence varied greatly between the nurseries (Fig. 4a), ranging from less than 5% to 100% of sampled fish. More than half of the individuals with nephrocalcinosis had mild changes in the kidney tissues (68%, Fig. 4a). The total proportion of fish with nephrocalcinosis was 45% in the nurseries. Among fish that were sampled one month after transfer to sea, the prevalence of nephrocalcinosis decreased to 18%. In fish groups with primarily mild degrees of nephrocalcinosis in the nursery, the prevalence had decreased (30% vs. 11%),

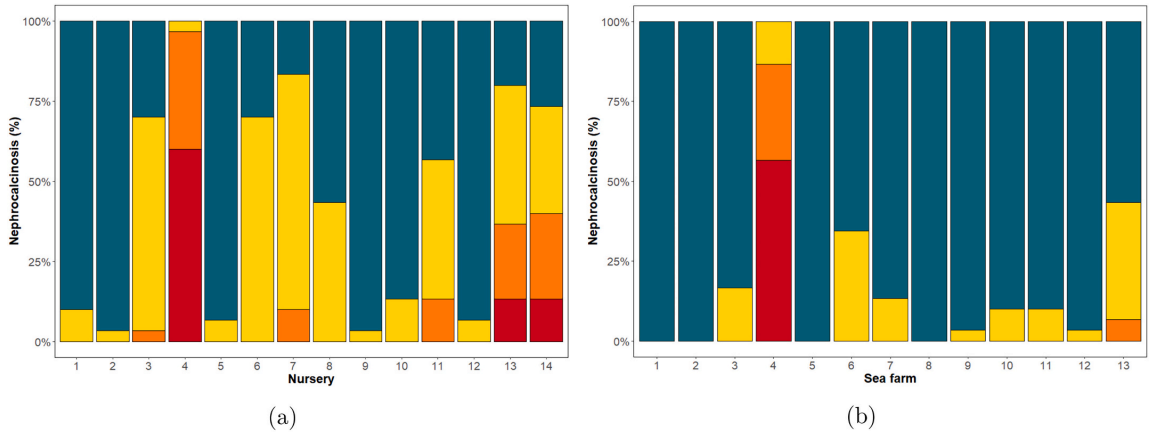

Fig. 4. Nephrocalcinosis in a) nurseries in Mid Norway($n = 420$) and b) receiving sea farm ($n = 390$). Yellow colour indicates mild changes, orange shows moderate changes and red indicates severe changes in the kidney tissue as found from histopathological examination. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

while in the groups with severe nephrocalcinosis the changes in the kidney persisted (6% vs. 4%, Fig. 4b).

3.3. Characterization of mineral deposits

A total of 69 samples of deposits were analysed by FTIR. The results revealed the presence of five different minerals: amorphous carbonate apatite (amCAP), struvite, brushite, whitlockite and newberyite. Amorphous carbonate apatite (amCAP) was the most prevalent mineral and was found in all but one of the mineral samples (Table 2). Struvite, brushite, whitlockite and newberyite were found in different combinations together with amCAP. From half of the nurseries only amCAP was found, while the remaining had different combinations of mixed minerals.

3.4. Plasma chemistry

A considerable proportion of fish affected by nephrocalcinosis showed plasma chemistry that differed from unaffected fish in several of the analysed parameters, with elevated calcium and magnesium being the most predominant changes (Table 3). A notable proportion of fish affected by nephrocalcinosis also displayed elevated levels of AST, inorganic phosphate, glucose and lactate, and lower levels of sodium and chloride, with reduced osmolality.

Smolts with severe changes in the kidney displayed significantly higher concentrations of AST, calcium, creatinine, glucose, magnesium, Na/K-ratio, inorganic phosphate, and urea. While plasma concentrations of chloride, potassium and sodium were significantly lower compared to the healthy group (Fig. 5). The plasma chemistry of fish with a moderate amount of nephrocalcinosis showed significantly higher concentrations of AST, calcium, magnesium, NA/K-ratio and inorganic phosphate, while chloride and potassium were significantly lower in this group compared to the healthy group (Fig. 5). The group with mild changes in the kidney had significantly higher concentrations of AST, calcium, glucose, lactate, magnesium, Na/K-ratio, inorganic phosphate and urea compared to the healthy group (Fig. 5).

Table 2
Mineral complexes found in Atlantic salmon reared in commercial nurseries in Mid-Norway ($n = 69$).

Mineral complex	Chemical formula	Number of samples
Amorphous carbonate apatite	$Ca_{10}(PO_4)_6CO_3$	68
Struvite	$MgNH_4PO_4$ x 6 H_2O	6
Brushite	$CaHPO_4$ x H_2O	7
Whitlockite	$MgCa_8(PO_4)_6$	10
Newberyite	$MgHPO_4$ x 3 H_2O	8

Table 3
Normal intervals of selected plasma chemistry parameters obtained from 229 healthy Atlantic salmon, with a comparison to data collected from salmon affected by nephrocalcinosis.

Parameter	Normal interval	Nephrocalcinosis		
		N	Higher	Lower
ALKP(U/L)	66–667	182	16 (9%)	7 (4%)
ALT (U/L)	10–38	101	14 (14%)	0 (–)
AST (U/L)	22–729	185	41 (22%)	1 (1%)
Creatinine (U/L)	9–80	119	15 (13%)	9 (8%)
Calcium (mmol/L)	2.1–3.4	186	69 (37%)	15 (8%)
Magnesium (mmol/L)	0.7–1.5	185	71 (38%)	7 (4%)
Phosphate (mmol/L)	2.4–8.1	184	29 (16%)	5 (3%)
Sodium (mmol/L)	149–169	186	9 (5%)	34 (18%)
Potassium (mmol/L)	1.5–4.9	182	5 (3%)	2 (1%)
Na/K	30–103	182	7 (4%)	4 (2%)
Chloride (mmol/L)	113–137	186	5 (3%)	34 (18%)
Osmolality (mmol/kg)	305–343	178	7 (4%)	30 (17%)
Glucose (mmol/L)	3.7–8.7	184	50 (27%)	2 (1%)
Lactate (mmol/L)	2.1–10.1	177	27 (15%)	2 (1%)
Urea (mmol/L)	0.6–1.7	182	23 (13%)	0 (–)

The comparison is shown as number and percentage of fish having a value higher or lower than the normal interval, out of the total number of valid samples (N).

4. Discussion

Nephrocalcinosis has in later years emerged as a major disease complex in Atlantic salmon reared in fresh water in Norway. To identify early markers, non-lethal diagnostic methods are important to reveal risk factors and support disease prevention. Further, determination of the composition of the kidney stones can provide clues about causation and potential ways to prevent stone formation. In the present study, histopathology, plasma chemistry and mineral deposit analysis were employed to get more insight into this disease in farmed Atlantic salmon.

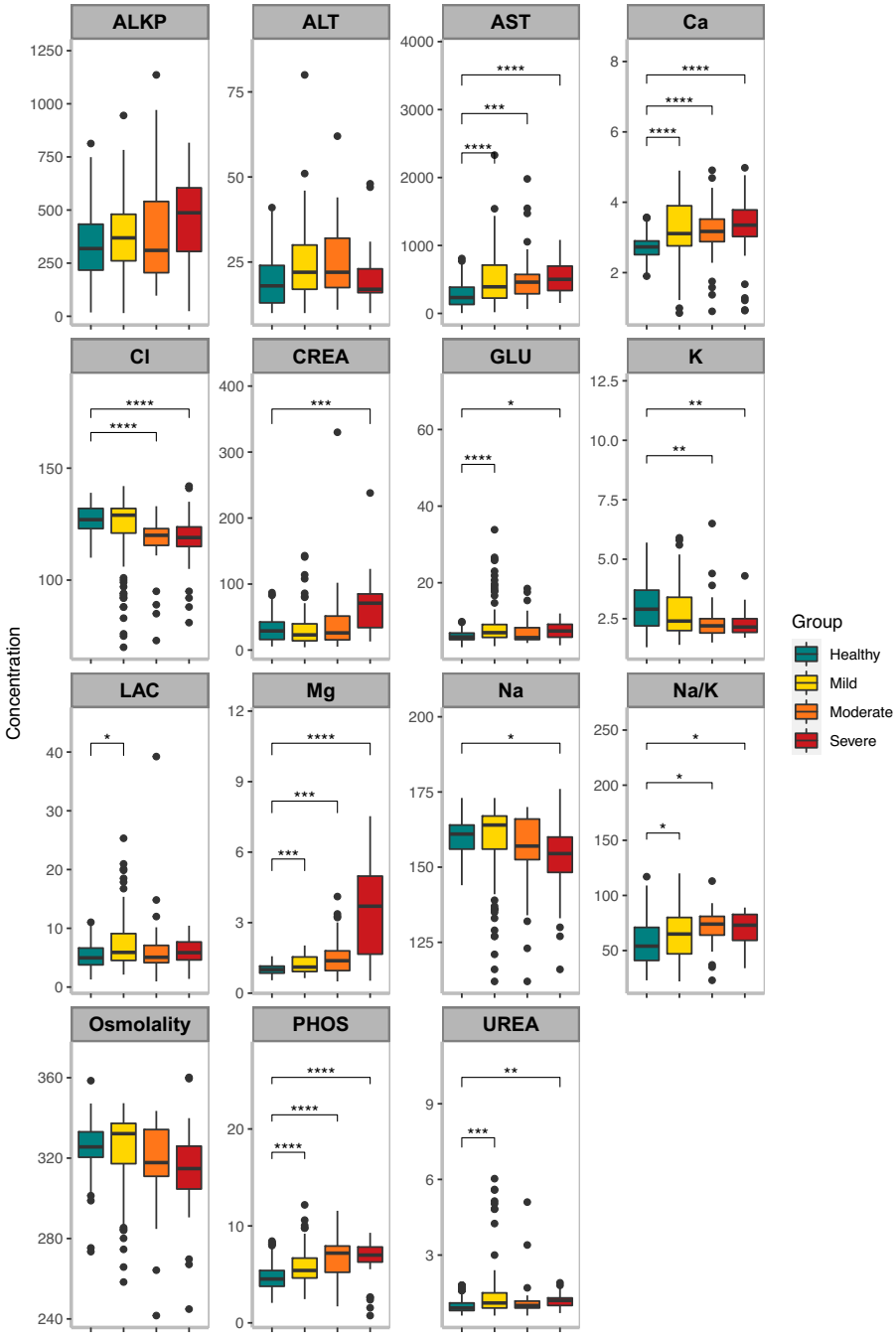

Fig. 5. Plasma chemistry parameters in Atlantic salmon with different severity of nephrocalcinosis (mild, moderate and severe, as assessed from histological evaluation), compared to healthy individuals as defined in Table 3. Significant differences denoted with */**/***/**** ($p<0.05/0.01/0.001/0.0001$). Measured parameters: Alkaline phosphatase (ALKP, U/L), Alanine aminotransferase (ALT, U/L), Aspartate aminotransferase (AST, U/L), Calcium (Ca, mmol/L), Chloride (Cl, mmol/L), Creatinine (CREA, μmol/L), Glucose (GLU, mmol/L), Potassium (K, mmol/L), Lactate (LAC, mmol/L), Magnesium (Mg, mmol/L), Sodium (Na, mmol/L), Osmolality (mmol/kg), Phosphate (PHOS, mmol/L) and Urea (UREA, mmol/L).

4.1. Methodology

Histopathology was used to assess the severity of tissue lesions and a weighed scoring system was developed for a more systematic approach for semi-quantification of the disease progression. The criteria and scoring method were designed to describe the changes in the kidney according to the known principles of pathology. The weighting factor was arbitrarily increased by one for each consecutive category to make sure that the more severe tissue-changes with increasing category number, resulted in higher contribution on the overall score. One limitation of the method is that the amount of tissue damage and the amount of deposits found in the histopathological sections do not always correlate. There are two main reasons for this: (1) the histopathological section represents a two-dimensional picture of a relatively large organ and there might be deposits in a plane that is not included in the section and (2) nephrocalcinosis in fish can heal (pers. obs.) and thus the chronic changes could remain whilst deposits could be reduced in quantity. Furthermore, primary and secondary causes of pathological changes in the kidney are challenging to differentiate histologically, as secondary changes caused by increasing inflammatory responses could be a result of chronic irritation due to nephrocalcinosis or other pathological conditions like Haemorrhagic Smolt Syndrome (HSS, pers. obs.). The accuracy of grading and the weighted scoring method is so far untested, but observations made in this study support the relevance for a weighed scoring, fish affected by nephrocalcinosis exhibited a wide range of histopathological changes. Fish affected by severe forms of nephrocalcinosis exhibited an almost complete loss of kidney structure while fish affected by the mildest forms of nephrocalcinosis exhibited at most negligible tissue damage.

Even though our results show that histopathology is a good diagnostic tool for nephrocalcinosis, non-lethal methods or early markers should be developed for a better monitoring of the disease. Blood chemistry analysis is often used as a diagnostic tool in several terrestrial species, but to our knowledge this methodology is infrequently used in farmed Atlantic salmon smolt. This is probably due to the lack of reference intervals for plasma chemistry in healthy individuals in different life stages, making it difficult to detect abnormalities. However, it would be advantageous if this method could be applied to farmed salmon as it would allow for rapid diagnostics without requiring euthanasia.

In this study, we used blood samples collected from 229 fish from 12 different nurseries to establish reference intervals for plasma concentrations for Atlantic salmon smolt reared in freshwater. Reference intervals were calculated following the guidelines from The American Society for Veterinary Clinical Pathology (ASVCP) (Friedrichs et al., 2012). Although of a similar magnitude, the normal intervals established in this study were wider than reference values previously reported by (Sandnes et al., 1988) for 10–20 adult Atlantic salmon from a single sea farm in Norway (Sandnes et al., 1988). One explanation may be that our data included a larger number of specimens as well as several facilities. Matsche et al. (2014) found statistical differences in plasma chemistry parameters in Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus*) from different production conditions. The variations in water chemistry and feeds between salmon facilities in our study may therefore have influenced the plasma chemistry. The wider interval of the plasma parameters may alternatively be linked to the time of sampling that coincided with the end of parr-smolt transformation in the nurseries (3905 $\pm$ 537 day degrees from roe to sampling point). Parr-smolt transformation is a complex process that results in a changed physiology in salmon (Folmar and Dickhoff, 1980), and is unlikely to be 100% synchronised in a population, causing differing physiology among the sampled fish. Still, there was no correlation between the different plasma parameters and weight (see Table A3 in supplementary material), and only minor correlation between plasma concentration of AST and gender (see Table A4 in supplementary material). We therefore believe that the reference intervals we propose are relevant for Atlantic salmon undergoing smoltification (60–210 g) in nurseries with rearing conditions in accordance with Norwegian regulations.

4.2. Nephrocalcinosis

The majority of salmon affected by nephrocalcinosis displayed mild changes in the kidney, characterized by deposits in renal collecting ducts and excretory ducts. Histopathological analyses showed substantial accumulation of mineral deposits in kidney tissues of fish affected by nephrocalcinosis. The deposits were mainly identified as amCAP, a calcium-dominated mineral. This is in line with Béland et al. (2020) which stated that calcium phosphate and struvite calculi appears to be overrepresented in fish, such as rainbow trout *Oncorhynchus mykiss* (Bjerknes et al., 1994; Gillespie and Evans, 1979; Smart et al., 1979), wolffish *Anarhichas lupus* (Béland et al., 2020) and southern flounder *Paralichthys lethostigma* (Applegate et al., 2016). Amorphous carbonate apatite indicates rapid precipitation. The formation of carbonate apatite (CAP) begins at pH $\geq$ 6.8, with an increasing ability to aggregate with increasing pH (Olszynski et al., 2015). The pH of the urine was not measured in this study, but Roy and Lall (2004) determined that normal values for urine pH in Atlantic salmon, are around 7.5 (Roy and Lall, 2004). Considering that amCAP and CAP have similar chemical properties, the normal high urine pH of salmon may predispose it for calcium phosphate precipitation in the kidney. In humans CAP is a constituent in about 40% of all stones (Kravdal et al., 2019). They are linked to hypercalcemia, hyperparathyroidism, distal renal tubular acidosis and urinary tract infections (Daudon and Jungers, 2012). In domestic animals calcium phosphate stones are quite rare (Osborne et al., 2009). When observed they are linked to hypercalcemia, hyperparathyroidism, hypervitaminosis D, and dystrophic and ectopic mineralization of vital tissues (Osborne et al., 1995). The underlying mechanisms of nephrocalcinosis in fish do not seem to relate to this, as we did not observe dystrophic or ectopic mineralization of vital tissues in our study. In addition, Tsertou et al. (2020) and Hilton and Ferguson (1982) did not find that excess of vitamin D3 could be related to the incidence of nephrocalcinosis in meagre (*Argyrosomus regius*) or rainbow trout (*Oncorhynshus mykiss*). However, nephrocalcinosis in salmon could be related to hypercalcemia as it was observed in 37% of salmon affected by nephrocalcinosis, and mean plasma concentrations of Ca^{2+} were significantly higher in salmon affected by nephrocalcinosis compared to healthy fish. In addition, the proportion of fish with hypercalcemia increased with increasing severity of nephrocalcinosis. It is not possible to determine whether the observed hypercalcaemia is a cause of the development of nephrocalcinosis or a consequence of nephrocalcinosis based on our data, and this should be investigated further.

The proportion of fish with nephrocalcinosis decreased after transfer to sea in fish with mild forms, which is in accordance with earlier observations (Fivelstad et al., 2003, 1999) This may indicate that mild changes in the kidney are reversible after the transfer to sea. The mechanisms involved in this process are yet unknown and should be considered in future studies. Even though it appears that mild forms of nephrocalcinosis in the fresh-water facility did not adversely affect survival after transfer to sea, it is likely that nephrocalcinosis negatively affects fish welfare. Our data shows that fish with mild changes display, in addition to elevated calcium, increased phosphate, AST, lactate and glucose concentrations in plasma, which are all signs of decreased welfare. AST is an enzyme which is considered a good indicator for tissue damage in fish (Li et al., 2011; Peres et al., 2015; Wagner and Congleton, 2004). Elevated plasma calcium and phosphate points to a disturbed regulation of homeostasis (Vielma and Lall, 1998) and elevated plasma glucose and lactate levels are linked to secondary stress responses (Barton and Iwama, 1991; Iversen and Eliassen, 2009; Iversen et al., 2009, 1998) and metabolic stress in salmon (Li et al., 2011).

Fish that were affected by moderate and severe forms of nephrocalcinosis exhibited much higher concentrations of magnesium in their plasma compared to healthy fish. The affected fish were diagnosed

with severe changes in the kidney, also described as pathological changes in glomeruli and interstitial tissue with extensive epithelial damage in the tubules. The primary site of magnesium excretion in fish is the kidney (Bijvelds et al., 1998) and elevated concentration of plasma magnesium has previously been linked to kidney damage (Nieves-Puigdoller et al., 2007; Singh et al., 2002). Fish with severe forms of nephrocalcinosis also displayed elevated plasma levels of AST indicating once again tissue damage, as well as significantly lower concentrations of potassium, sodium and chloride, which are considered as signals for reduced osmoregulatory capacity (Carey and McCormick, 1998; McDonald and Milligan, 1997).

Fish health personnel at the sea farms included in this study reported nephrocalcinosis to be a contributor to the registered mortality in the groups with severe nephrocalcinosis. To the authors knowledge there is no scientific publication that has investigated the potential direct mortality caused by nephrocalcinosis in salmon, but Jelmert et al. (1995) reported mass mortality in cultured Atlantic halibut larvae linked to the same disease. Unlike a freshwater environment, seawater is hyperosmotic and causes a net water loss by osmosis in fish (Beyenbach, 2004). The transition from freshwater to seawater is a demanding process for anadrome species like salmon and it requires drastic changes in the physiology of several organs such as the kidney (Takvam et al., 2021). Fish affected by severe nephrocalcinosis exhibit a loss of normal kidney structure with vast amounts of calcium deposits and extensive damage in glomeruli and interstitial tissue. These individuals are thus likely to have a highly reduced kidney function, which probably makes adaption to seawater extremely challenging for the organ. This may, in turn, lead to increased mortality after transfer to sea, either directly as a loss of osmoregulatory function in the kidney or indirectly by increased susceptibility to stress and secondary infections by bacteria.

This study revealed that plasma chemistry analyses is not adequate to diagnose nephrocalcinosis. Normal blood chemistry values do not necessarily indicate absence of nephrocalcinosis, since a notable proportion of the fish with nephrocalcinosis displayed plasma chemistry values within the normal intervals. On the contrary plasma chemistry values can be used as preliminary diagnosis for the disease, as changes in calcium, magnesium, glucose and AST were observed in a pronounced part of the fish affected by nephrocalcinosis.The preliminary diagnosis of nephrocalcinosis should thereafter encourage subsequent investigations with histopathological methods to confirm the diagnosis. In this study we investigated 15 different blood parameters and we can't disregard that other components could be relevant for diagnosis of nephrocalcinosis.

5. Conclusion

This study clearly showed that nephrocalcinosis is a common production disorder in farmed Atlantic Salmon in nurseries in Mid-Norway. The fish studied exhibited a broad range of severity, ranging from sparse amounts of deposits in tubules and collecting ducts to pathological changes in glomeruli and the interstitial tissue. Although the majority of the examined fish were only mildly affected, our results revealed that nephrocalcinosis is a welfare challenge. A considerable percentage of the fish affected by nephrocalcinosis had altered plasma chemistry, indicating stress and osmoregulatory disorders. Even though it is not possible to diagnose nephrocalcinosis with a blood sample at this stage, elevated plasma levels of magnesium, calcium, glucose and AST can be used as indicators for disturbed physiology that may be related to nephrocalcinosis. Routine blood samples may therefore be used as a relatively easy detection tool, where detection needs to be followed up by autopsy and histopathology, to confirm a suspicion of nephrocalcinosis.

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Author contributions

CK and LB were responsible for the study conception and design, and funding acquisition. CK carried out the field work and was responsible for data analysis and visualisation. MKM analysed the mineral deposits and AKR and ASD performed the histopathological assessments. CK wrote the manuscript with support from AKR, ASD and LB. LB, KJKA and REO supervised the work and aided in interpreting the results.

Declaration of Competing Interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2022.738104.

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PAPER II

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RESEARCH ARTICLE

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Gene expression patterns in Atlantic salmon (*Salmo salar*) with severe nephrocalcinosis

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Abstract

Nephrocalcinosis is a common disorder in farmed Atlantic salmon, but the consequences for the fish physiology are not well understood. We performed a transcriptome study in kidneys of Atlantic salmon (*Salmo salar*) smolts without and with severe chronic nephrocalcinosis (NC). The study revealed that numerous genes are differentially expressed in fish with NC compared with healthy salmon. The most evident changes in gene expression patterns in the NC group were a massive downregulation of metabolism and energy production, upregulation of signalling pathways important for tissue repair and function maintenance and upregulation of inflammatory responses. Overall, the extensive tissue damage and the gene regulation responses that affect salmon with severe nephrocalcinosis are highly likely to have dramatic consequences on fish survival.

KEYWORDS
Atlantic salmon, kidney malfunction, nephrocalcinosis, RNA seq

1 | INTRODUCTION

Nephrocalcinosis is described as deposits of mineral salts within kidney tubules and collecting ducts (Bruno, 1996). Renal calcification may occur at molecular, microscopic or macroscopic levels in the kidney, such as tubular cells, interstitial tissue or within the tubular lumen that may lead to progressive amounts of renal damages (Sayer et al., 2004). In a previous study, we found that the renal deposits in Atlantic salmon mainly consisted of amorphous carbonate apatite (amCAP), a calcium-dominated mineral (Klykken et al., 2022).

The aetiology of nephrocalcinosis in fish is not known (Klosterhoff et al., 2015), but there is a consensus that the condition is most likely related to the intensive production conditions in aquaculture (Applegate et al., 2016; Béland et al., 2020; Bjerknes et al., 1994; Cavrois-Rogacki et al., 2021; Gillespie & Evans, 1979; Klosterhoff et al., 2015; Lewisch et al., 2013; Smart et al., 1979).

The kidney is one of three primary organs (kidney, gills and intestine) involved in osmoregulation in salmon (Talbot & Thorpe, 1992). The renal function of the anadromous salmon undergoes major transformations as the fish prepare to migrate from freshwater (FW) to seawater (SW) environments (Takvam et al., 2021). In the hyperosmotic environment of FW, one of the main tasks of the glomerular kidneys is to excrete excess water while reabsorbing solutes (McDonald, 2007). In contrast, to counteract the potential dehydration in SW, salmon drink ambient water containing large concentrations of divalent ions. The primary function of the kidney in SW is,

therefore, to excrete divalent ions in strongly reduced isotonic urine (Beyenbach, 2000).

Severe nephrocalcinosis with extensive damage to the kidney is likely to result in impaired renal function. This can have dramatic consequences for osmoregulatory capability like water export in FW and divalent ion excretion in SW, posing a threat to health and welfare of the salmon. In the aquaculture industry, there is a general consensus that the disease is linked to commercial production conditions, but little is known of the internal mechanisms of nephrocalcinosis. To explore these mechanisms, we applied whole transcriptome profiling of salmon kidneys with severe nephrocalcinosis and compared the gene expression patterns with healthy salmon kidneys.

Transcriptome profiling is a powerful tool used to expose expression patterns (Rani & Sharma, 2017). Variations in the transcriptome can be observed for physiological conditions, developmental stages and the external environment of an organism which makes transcriptome profiling a robust tool for examining the relationship between the genotype and phenotype (Chandhini & Kumar, 2019). In aquaculture, transcriptomics has previously been utilized for examining immunity, diseases and nutrition (Martin et al., 2016; Martin & Król, 2017; Sudhagar et al., 2018; Ye et al., 2018) as well as detection of molecular markers (Chandhini & Kumar, 2019).

We hypothesize that severe nephrocalcinosis results in a generally altered physiology with differential gene regulation responses. By studying the effects of nephrocalcinosis on fish physiology and gene expression patterns, we aim to provide new insight into the mechanisms of the disease.

2 | MATERIALS AND METHODS

2.1 | Data sampling

From a previous study with 420 farmed Atlantic salmon, which were analysed for nephrocalcinosis (Klykken et al., 2022), 16 were chosen according to the criteria of being either healthy (Ctrl, $N = 8$) or severely affected by nephrocalcinosis (NC, $N = 8$). The fish were smolts of similar sizes with no other pathological findings. The fish were collected from two different commercial salmon nurseries with production conditions complying with the Norwegian legislation (Akvakulturdriftsforskriften, 2008). The fish were fed until sampling, and they were killed with an overdose of Benzoak VET (200–400 mg/L) followed by a sharp blow to the head according to Norwegian legislation (Akvakulturdriftsforskriften, 2008). Size measurements of individual fish included round body weight (W) in g (±1 g), fork length (L) (±0.5 cm), and condition factor: $CF = 100(W/L^3)$.

2.2 | Histopathology

Tissues from the mid-kidney were sampled from all individuals for histopathological analysis of nephrocalcinosis. The kidney tissues were fixed in 4% formaldehyde solution, embedded in paraffin wax and routinely processed (Suvarna et al., 2019). All sections were stained by haematoxylin and eosin. A selection of sections in paraffin was stained with von Kossa stain for visualization of calcium

TABLE 1 Histopathological nephrocalcinosis score

Severity	1	2	3
Category 1			
Presence of deposits	Sparse amounts in collecting ducts and ureters close to absence in tubules and affects <10% of the excretory system	Moderate amounts in collecting ducts and ureters sparse amounts in tubules and affects between 10% and 50% of the excretory system	Extensive quantities in ureters, collecting ducts and tubules and affecting more than 50% of the excretory system
Category 2			
Epithelial degeneration and/or necrosis	Affects <10% of tubules and collecting ducts	Affects between 10% and 50% of tubules and collecting ducts	Affects more than 50% of tubules and collecting ducts
Category 3			
Pathological changes in the glomeruli	Dilatation of the glomerular space (urine stagnation) and fibrosis/thickening of the parietal Bowman's capsule, changes in <10% of the glomeruli	Dilatation/thickening of the parietal Bowman's capsule, peri-glomerular fibrosis—changes in between 10% and 50% of glomeruli	Dilatation, thickening of the parietal Bowman's capsule, peri-glomerular fibrosis, changes in over 50% of glomeruli
Category 4			
Pathological changes in the interstitial tissue	Affects <10% of interstitial tissue	Affects between 10% and 50% of interstitial tissue	Affects more than 50% of interstitial tissue.

Note: The scores are weighted based on the effect the various changes are believed to have on the development of the disease and the time it will take to heal the condition.

deposits (Rungby et al., 1993). The histopathological diagnosis of nephrocalcinosis was defined as the presence of amorphous (structureless), basophilic deposits in tubules, collecting ducts and excretory ducts. The severity of deposits and tissue damage was evaluated according to the score given in Klykken et al. (2022) (Table 1). Total nephrocalcinosis score is calculated as:

$$\sum_{n=1}^{4} C_n \times S_{C_n}$$

Where *C* is the category, *S* is the severity and *n* is the category number.

In the nephrocalcinosis score, overall scores 1 to 10 were generally considered mild changes, scores 11 to 20 were considered moderate changes, and scores greater than 20 were regarded as severe changes.

2.3 | Blood collection and determination of plasma chemistry

Vacutainer tubes (Becton-Dickinson) with lithium heparin as anticoagulant were used to collect blood from the caudal vein, immediately after euthanasia. After thorough mixing, the samples were centrifuged at 13,500 rpm for 5 min (VWR Mikrostar 12, 12×1.5/2.0 ml) and the plasma was transferred to Eppendorf tubes and kept frozen (minimum −20 C) until analysis.

FIGURE 1 Kidney with extensive amounts of mineral deposits in ureters (arrow) and bladder (star)

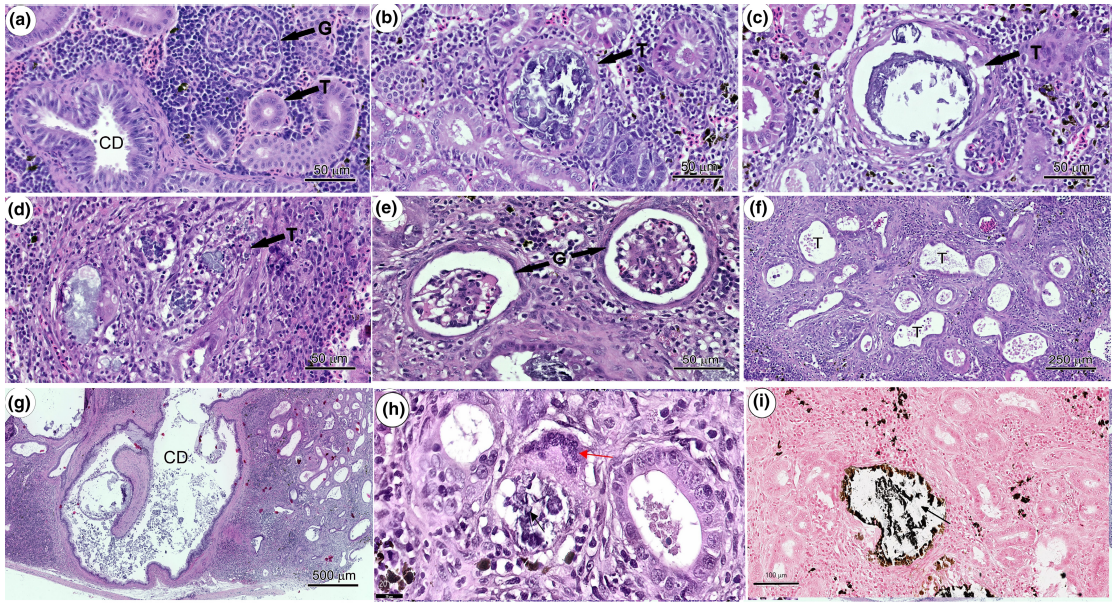

FIGURE 2 Histopathological changes associated with severe nephrocalcinosis. (a) Normal tissue showing a healthy glomerulus *(G)*, tubulus *(T)* and collecting duct *(CD)*. (b) Moderate lesion in a tubule, with marked degeneration and necrosis of the epithelium and rich amounts of deposits in the lumen. (c) Extensive, chronic lesions in a tubule, with complete loss of epithelium, fibrosis of the basal membrane, and dilatation of the lumen-containing deposits. (d) Advanced, chronic lesion with complete destruction of the tubular structure including the basal membrane, with massive inflammation in the interstitium. (e) Chronic changes in the glomeruli, with dilatation of the Bowman's space, fibrosis and thickening of the parietal layer of the Bowman capsule, peri-glomerular fibrosis and moderate glomerulitis. (f) Chronic changes, with degeneration of epithelium and dilatation of tubules, surrounded by extensive fibrosis replacing normal interstitial tissue. (g) Chronic changes, with extensive dilatation of a collecting duct, degeneration and necrosis of epithelium and the presence of deposits together with necrotic cells in the lumen. (h) Langhans giant cells (red arrows). (i) von Kossa stain of kidney tissue with deposits and tissue reaction associated with nephrocalcinosis. Black staining (arrows) indicative of the presence of calcium salt, surrounded by a granulomatous inflammatory process

The following parameters were measured at the laboratory of Aqua Kompetanse using an automated dry chemistry analyser (CatalystOne, IDEXX Laboratories, Westbrook, ME, [Boes et al., 2018]): alkaline phosphatase (ALKP), aspartate aminotransferase (AST), creatinine (CREA), calcium (Ca), sodium (Na), potassium (K), chloride (Cl), magnesium (Mg), inorganic phosphate (PHOS), glucose (GLU) and lactate (LAC). Each of the assays used a standard kit developed for the automated analyser (CatalystOne, IDEXX Laboratories, Westbrook, ME).

The plasma occasionally showed activities for inorganic phosphate exceeding the instrument ranges and sporadically for other parameters: in these cases, a physiological saline solution (0.9% NaCl) was used to dilute the samples (1:4).

2.4 | Gene expression patterns by mRNA sequencing

Mid-kidney tissue was isolated and immediately transferred into tubes containing RNAlater ©(Life technologies) and stored in 4 °C for 24 h before storage at −20 °C until analysis.

The samples were processed according to (Løvmo et al., 2021). Total RNA was extracted from the mid-kidney of 8 fish with severe nephrocalcinosis and 8 healthy fish, a total of 16 samples. mRNA extraction was done using a RNeasy Plus Universal Mini kit (Qiagen) according to the manufacturer's instruction. RNA concentrations were measured using a Nanodrop 8000 (Thermo Scientific). RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). All 16 samples passed the quality check (RIN >7). cDNA libraries were prepared using TruSeq Stranded mRNA Sample prep HS protocol (Illumina) according to the manufacturer's instruction, with selection for 500 bp fragments. The cDNA libraries were sent to Genomics Core Facility at the Norwegian University of Science and Technology (NTNU) for sequencing. Libraries were quantified by qPCR using the KAPA SYBR FAST library quantification kit for Illumina Genome Analyser (KAPA Biosystems). A 2.5 nM solution of the sequencing library pool was subjected to cluster generation on a HiSeq4000 flow cells by the cBot instrument (Illumina, Inc.). Paired-end sequencing was performed for 2×75 cycles on a HiSeq4000 instrument (Illumina, Inc.), according to the manufacturer's instructions, producing 75 bp reads. Base calling was done on the HiSeq4000 instrument by RTA 2.7.7. FASTQ files were generated using bcl2fastq2 Conversion Software V2.20.0422 (Illumina, Inc.).

2.4.1 | Quality control

Sequence reads were de-multiplexed and converted from BCL to fastq file format using bcl2fastq2 conversion software V2.20.0422 (Illumina, Inc.). Reads were mapped to the Atlantic Salmon reference genome (ICSASG_v2. 6-10-2016) using HISAT2 v2.1.0 with default parameters (Kim et al., 2015). Gene expression levels were based on read counts per gene and were quantified from mapping results using feature Counts v1.6.5 (Liao et al., 2014). Gene regions on the ICSASG_v2 reference genome were defined using RefSeq annotation data and metadata. A count table of read counts per gene for each sample was used as the foundation for comparative analysis of gene expression. The table was imported into R version 4.0.5 (Team, 2017) where all subsequent downstream analyses were completed, including differential expression analysis, pathway enrichment and functional annotation. Sample variance and clustering, including outlier detection, was assessed by generating density plots, PCA plots, heat maps (Figure S1) and dendrograms using base R tools. Differential expression (DE) analysis was completed using the DESeq2 package (Love et al., 2014). Identification of DE genes by DESeq2 was done using the following statistical steps: normalization completed by multiplying read counts by per-sample size factors, which were generated by dividing the read counts per sample by the geometric mean for each gene across all samples. Then, genewise dispersions were estimated and shrunk. These shrunken estimates are fit to a negative binomial generalized linear model (GLM), estimating expression strength per gene, by group. A Wald test was used to test the significance of expression strength between groups, generating p values for each gene. Finally, the false discovery rate (FDR) of p values was estimated using the Benjamini and Hochberg method (Benjamini &

TABLE 2 Body weight, length, condition factor and blood chemistry parameters of nephrocalcinosis-affected (NC) salmon compared with healthy fish (Ctrl)

Parameter	NC	Ctrl
Number of fish	8	8
Body weight (g)	279 ± 52	249 ± 12
Length (cm)	29 ± 2	28 ± 1
Condition factor	1.2 ± 0.1	1.1 ± 0.1
Enzymes		
Alkaline phosphatase (U/L)	370.8 ± 192.5*	669.1 ± 103.6
Aspartate aminotransferase (U/L)	476.8 ± 204.1*	247.4 ± 89.2
Creatinine (U/L)	84.9 ± 76.8	37.8 ± 16.3
Electrolytes		
Calcium (mmol/L)	3.7 ± 0.7*	2.7 ± 0.2
Magnesium (mmol/L)	5.2 ± 1.2*	1.0 ± 0.2
Phosphate (mmol/L)	7.1 ± 1.1*	4.5 ± 0.8
Sodium (mmol/L)	160.1 ± 12.0	162.1 ± 1.9
Potassium (mmol/L)	2.3 ± 0.5	3.1 ± 1.0
Chloride (mmol/L)	126.5 ± 12.1	127.5 ± 3.9
Metabolites		
Glucose (mmol/L)	9.4 ± 1.8*	6.5 ± 0.8
Lactate (mmol/L)	8.2 ± 1.2*	3.4 ± 0.8

Note: Significant differences between the groups are indicated with * ($p \leq .05$).

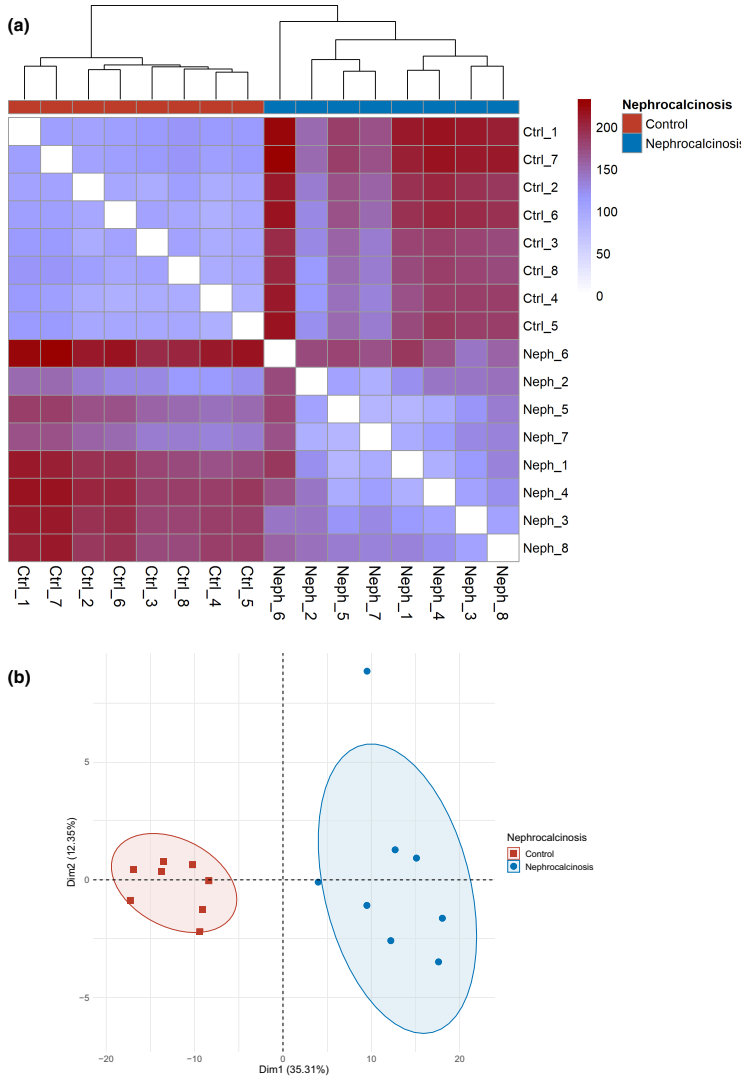

FIGURE 3 (a) Heatmap and dendrogram of variance stabilizing transformed (vst), normalized mean counts per sample. (b) PCA plot of transformed normalized mean counts per sample, nephrocalcinosis in blue and control group in red. Shaded areas represent 85% confidence interval (CI) of variance per treatment group

Hochberg, 1995). Significantly DE genes were identified as having an FDR adjusted p value of <.05.

2.5 | Statistical analysis

All statistical analyses were performed using R software 4.0.5 (R Core Team, 2017). Normality was tested with Shapiro–Wilks test, and non-normal distributed data were transformed using square root, cube root or Tukey's ladder. Magnesium concentration in plasma was not normally distributed. These samples were analysed using Wilcoxon rank-sum test. Blood chemistry parameters are presented as mean ± standard deviation. p values of ≤.05 were considered statistically significant.

3 | RESULTS

3.1 | Histopathology of kidney with nephrocalcinosis

There were extensive amounts of mineral deposits in ureters and bladder of fish affected by nephrocalcinosis (NC) (Figure 1). The kidneys of the affected fish were swollen and the tissue appeared pale compared with controls. All fish in the NC group (n = 8) were diagnosed with severe nephrocalcinosis with a score ranging from 26 to 30 (Table 1).

The NC group displayed extensive damage to the tubular wall, with complete loss of epithelium with fibrosis of the basal membrane and dilatation and fibrosis in the lumen-containing deposits (Figure 2b–g).

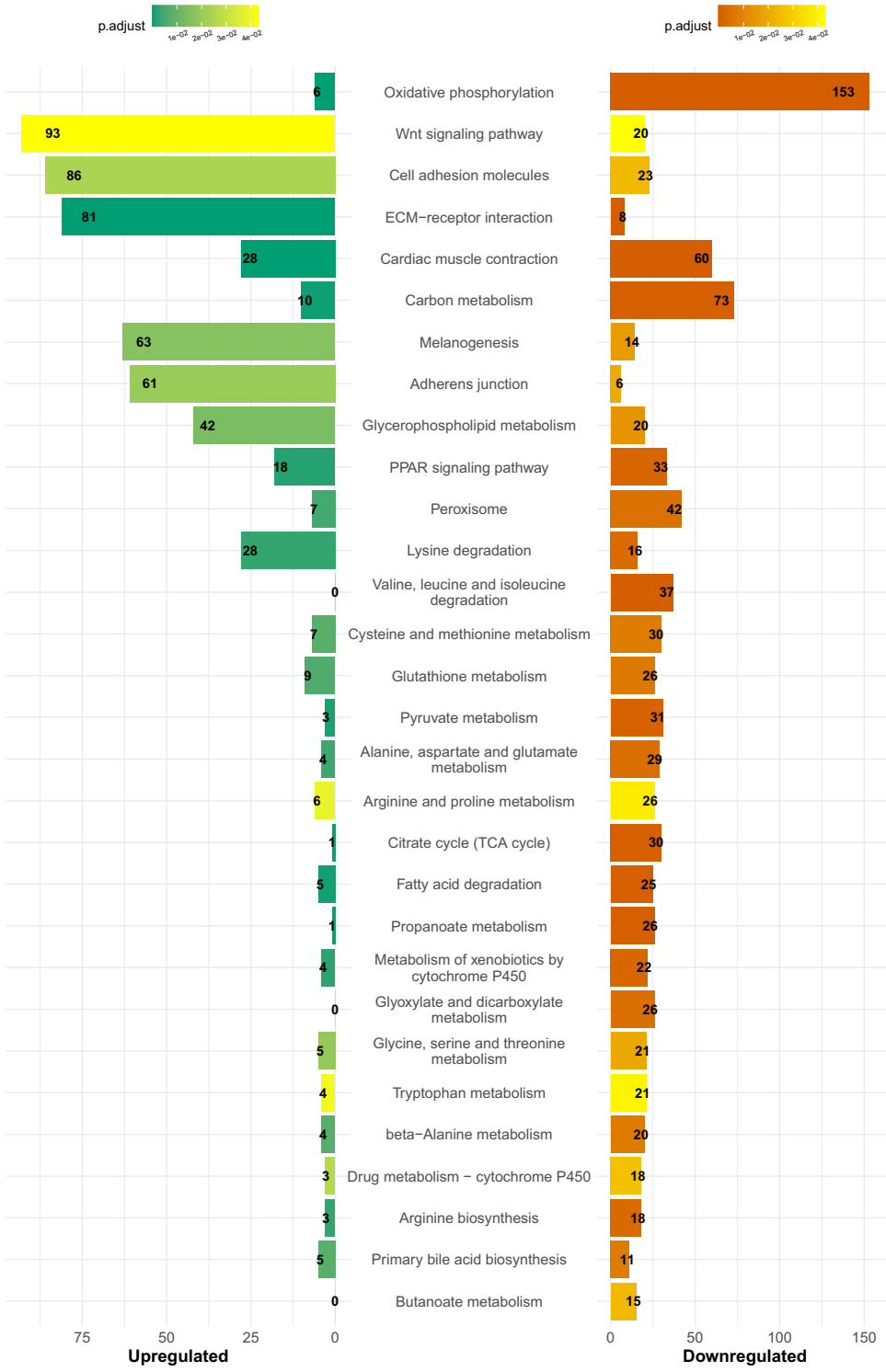

FIGURE 4 Mirrored bar plot of the number of upregulated and downregulated DE genes in each significantly (padj < 0.05) enriched KEGG pathway Ctrl Vs NC. Bars are shaded by adjusted *p* values per pathway

Acute interstitial inflammation and chronic interstitial fibrosis was also observed (Figure 2f). The surrounding interstitial tissue showed granulomatous inflammation with the presence of Langhans giant cells (Figure 2h) and chronic glomerulitis was also seen (Figure 2b). Von Kossa showed black staining of the tissue, indicating calcium salts in the deposits associated with nephrocalcinosis (Figure 2l).

3.2 | Blood chemistry

The blood chemistry parameters differed significantly between Ctrl and NC (Table 2). The NC group had significantly higher concentrations of aspartate aminotransferase (AST), calcium, magnesium, phosphate, glucose and lactate, whereas alkaline phosphatase (ALKP) was significantly lower. The variance in the NC group was considerably higher than in the Ctrl.

3.3 | Gene expression patterns

A total of 10133 genes displayed differential expression patterns in fish with severe nephrocalcinosis (NC) compared with healthy fish (Ctrl). 6970 of the DE genes were upregulated (log2fold change [LFC] >1) and 3163 were downregulated (LFC <−1).

The gene expression patterns of the two groups, Ctrl and NC, showed good separation as illustrated in the heatmap and PCA plot (Figure 3). The KEGG pathway enrichment analysis was used to identify pathways significantly regulated (enriched DEGs) (Figure 4 shows top 30). There were 31 significantly enriched KEGG pathways. Among these were 22 metabolic pathways, 4 pathways involved in environmental information processing, 2 pathways connected to cellular processes and 3 pathways belonging to organismal systems.

One of the most significant changes in fish affected with nephrocalcinosis was the general and apparently massive downregulation of many metabolic pathways related to ATP production, glycolysis and Krebs cycle activity. The oxidative phosphorylation and Krebs cycle appeared to be particularly affected with 153 and 30 genes, respectively, significantly downregulated (Figures 5 and 6, respectively). Many other processes feeding into these pathways were also downregulated such as peroxisomes, fatty acid and amino acid degradation/metabolism. Other notable pathways downregulated included the P450 xenobiotics. In addition, the glutathione metabolism pathway was mostly downregulated, with exception of ribonucleoside-diphosphate reductase which was upregulated. This

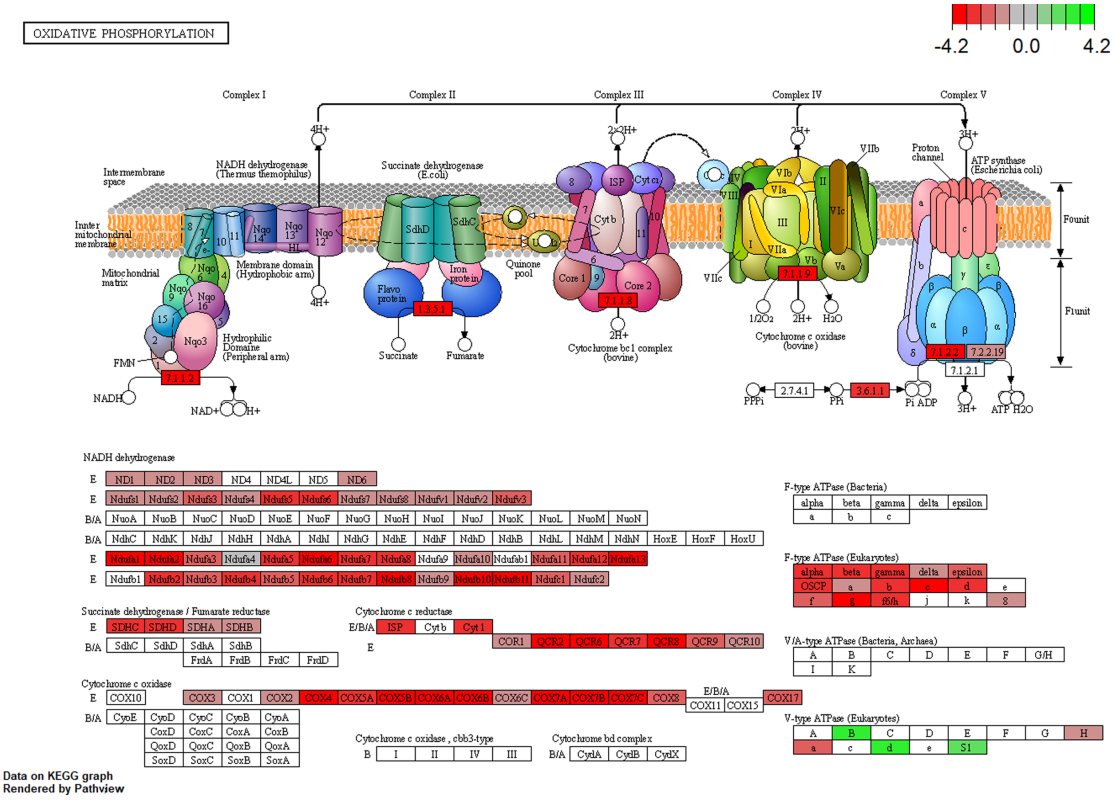

FIGURE 5 Pathway map sasa00190—oxidative phosphorylation. The protein EC number within each box is given in either green, red or white, indicating upregulation, downregulation or no change, respectively, with increased colour intensity indicating increased log2fold change

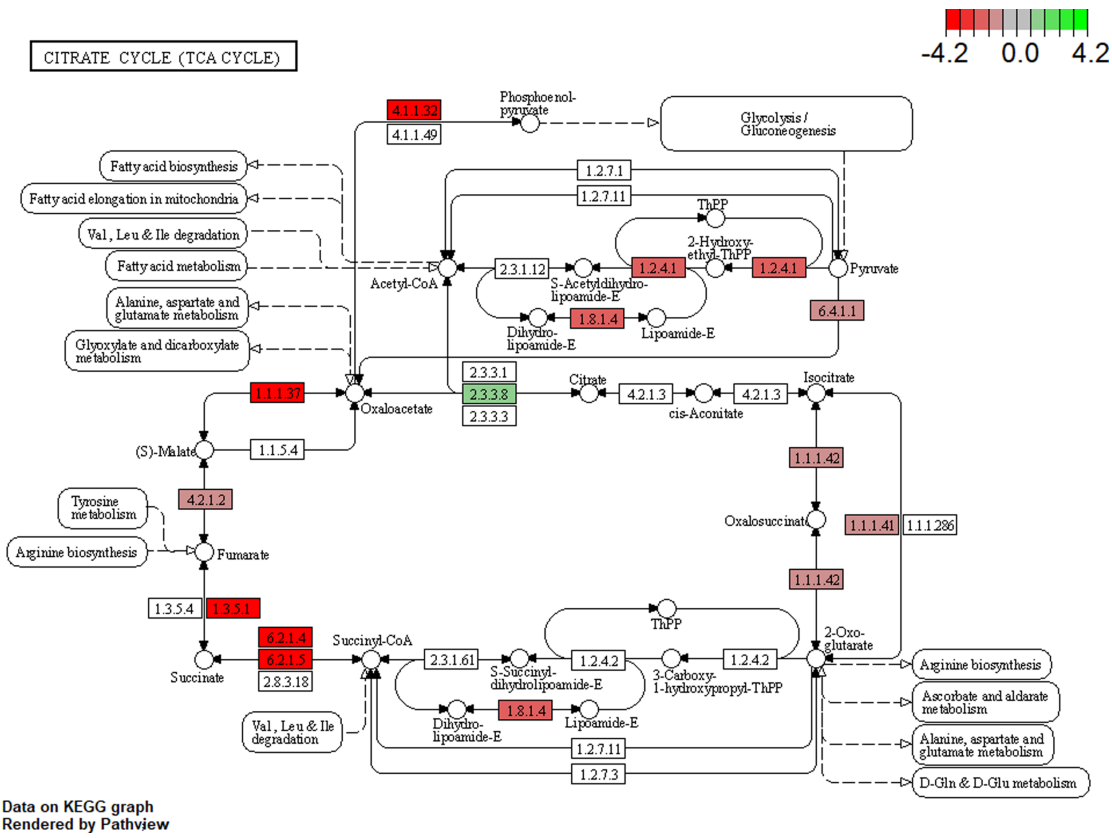

FIGURE 6 Pathway map sasa00020—citrate cycle. The protein EC number within each box is given in either green, red or white, indicating upregulation, downregulation or no change, respectively, with increased colour intensity indicating increased log2fold change

pathway is important for DNA and RNA repair, and the upregulation suggests increased repair requirement in the tissue.

Most gene pathways upregulated in fish with nephrocalcinosis were related to cell cycle and maintenance. All three Wnt signalling pathways were upregulated (93 genes) including the canonical Wnt pathway, the non-canonical planar cell polarity pathway and the non-canonical Wnt/calcium pathway. Adherence junctions were also strongly upregulated (61 genes) including transmembrane nectin and cadherin (Figure 7).

Upregulated genes downstream of nectin included many genes involved in cytoskeleton and cell shape regulation (PAR3, Src, Ponsin, Zo-1, alpha-actinin and IRSp53). Likewise, numerous cell adhesion molecules were also strongly upregulated in fish affected with nephrocalcinosis. These include many leucocyte proteins including major histocompatibility complex II (MHCII), CD22 (B cells), B7H3 and poliovirus receptor-related 2 (PVRL2) and 3. It was interesting to note that some members of the adaptive immune system like CD2 found on T and NK cells were downregulated. CD99 was upregulated, indicating the migration of immune cells to inflammation in the kidney tissue (Figure 8). There was also a notable upregulation of genes encoding for the junctional adhesion proteins B and C (junctional adhesion molecules 2 and 3, respectively, JAM2, JAM3), but not JAM1. Interestingly, the expression of another set of tight junctional proteins, claudins (claudin 2, 3, 10 and 14), was downregulated.

The signal transduction pathways were upregulated, as were the signalling molecules and interaction pathways, and in particular, the ECM-receptor interaction pathway was highly upregulated (Figure 9). Genes involved in cellular communication were also primarily upregulated.

Genes in the cardiac muscle contraction pathway were primarily downregulated (60 downregulated and 28 upregulated, Figure 10). Tropomyosin (TPM) was greatly upregulated in the NC group, pointing to cell structure maintenance and repair since TPM and myosin are important for cytoskeleton and cell migration. The ubiquinol-cytochrome and cytochrome c subunits were also downregulated. These molecules are part of oxidative phosphorylation and metabolism, which were distinctly downregulated.

Genes involved in lipid metabolism were both up- and downregulated, with the majority of pathways having a predominance of

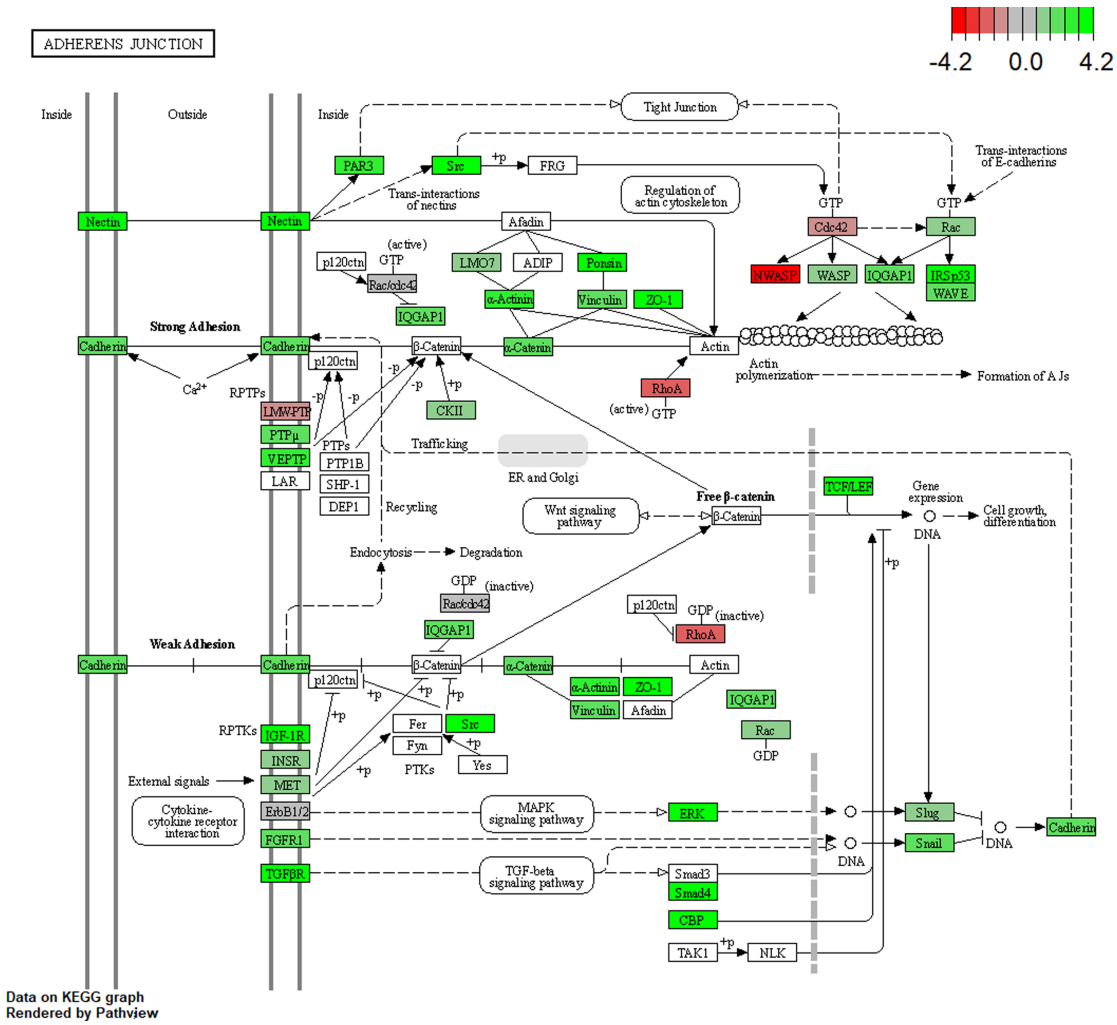

FIGURE 7 Pathway map sasa04520—adherence junctions. The protein EC number within each box is given in either green, red or white, indicating upregulation, downregulation or no change, respectively, with increased colour intensity indicating increased log2fold change

downregulated genes with the exceptions of fatty acid degradation and glycerophospholipid metabolism, which had more upregulated genes. Arachidonic acid metabolism had several upregulated genes. The increase in the prostaglandin endoperoxide synthase and prostacyclin synthase suggests an increased synthesis of prostacyclin, which is an efficient inhibitor of platelet activation and a vasodilator. The activity of PLA2 appeared to increase, suggesting increased turnover of membrane lipids. There was also an increased phospholipid synthesis, particularly through the Kennedy pathway, and towards phosphatidylethanolamine, choline and other phospholipids. Interestingly, the activity of acetylcholinesterase was also increased pointing to increased neuronal signalling (see Figure S1–S16 in Appendix).

4 | DISCUSSION

This is the first transcriptome study profiling the response of Atlantic salmon (*Salmo salar*) with chronic and severe nephrocalcinosis (NC). The study showed numerous genes and KEGG pathways being differentially expressed in salmon with nephrocalcinosis compared with healthy salmon (Ctrl). In order to gather a sufficient number of samples from healthy fish and NC fish, kidney samples had to be collected from two separate nurseries. We are aware that this is a scientific element of uncertainty in the study, as we cannot know to which level the different environments may have affected the gene expression patterns in the two groups. Despite this, we consider the

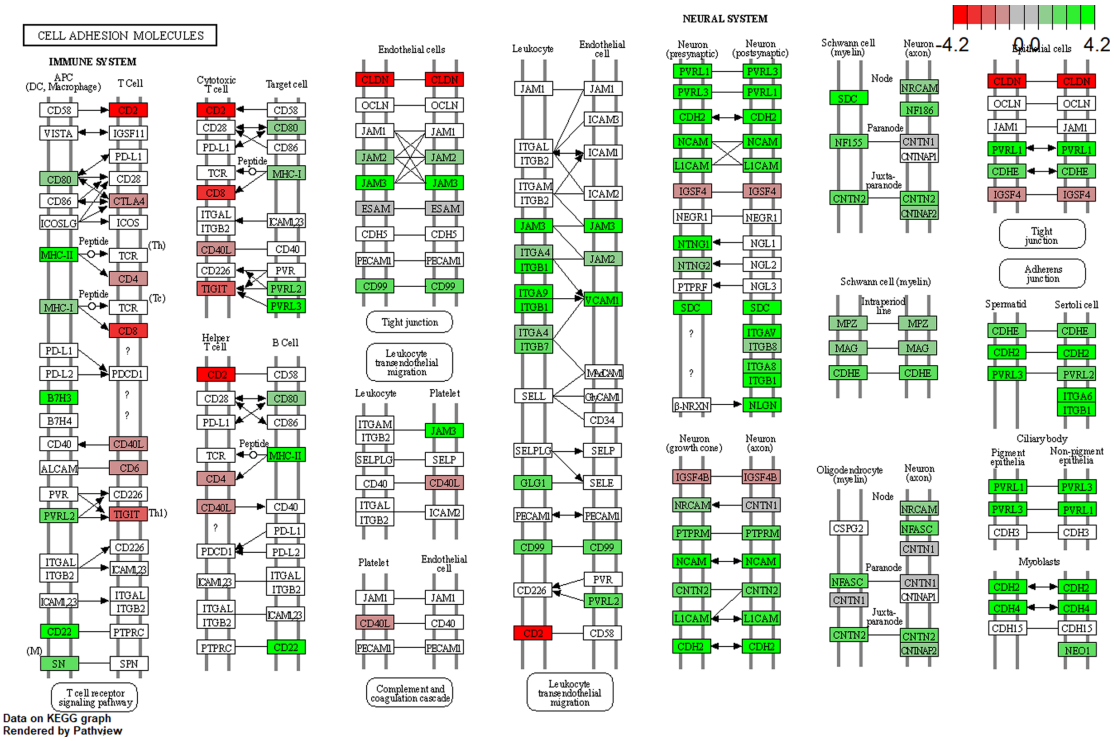

FIGURE 8 Pathway map sasa04514—cell adhesion molecules. The protein EC number within each box is given in either green, red or white, indicating upregulation, downregulation or no change, respectively, with increased colour intensity indicating increased log2fold change

study of value because we believe that the main differences in the transcriptome profiles are related to the pronounced tissue damage caused by nephrocalcinosis.

Histological examination of Atlantic salmon kidneys with severe NC revealed, extensive tissue damage similar to earlier observations (Harrison & Richards, 1979; Hicks et al., 1984; Klykken et al., 2022; Saraiva et al., 2016). The effects included changes in the glomeruli with dilatation of the glomerular space, fibrosis and thickening of the parietal layer of the Bowman's capsule and varying degree of perglomerular fibrosis and glomerulitis. The damages to glomeruli have been suggested, in parts, to be a result of urine stagnation (Docherty et al., 2006) caused by mineral deposits formed during the development of NC (Harrison & Richards, 1979). There was also a significant granulomatous inflammation with the formation of giant cells in the NC group that would have been a response to cell injury and persistent irritants (O'Regan & Berman, 2001; Sakai et al., 2012; Shah et al., 2017). Giant cells are typical for granulomatous inflammation and are formed through the fusion of epithelioid macrophages (Damjanov, 2009; Kumar et al., 2013). Granulomatous inflammation was also found in the interstitium where the mineral deposits formed within tubules appeared to penetrate the tubular wall to interact with the immune cells of the interstitium (Shavit et al., 2015). This infiltration of leucocytes is supported by the upregulation of transcriptomic upregulation in the WNT signalling and cell adhesion pathways in the NC group. These pathways are involved in immune cell maintenance and renewal (Patel et al., 2019), as well as immune response and inflammation. The cell adhesion pathway includes leukocyte transendothelial migration, which was upregulated in the NC group. Leukocyte transendothelial migration is a critical step in immune activation (Gao et al., 2017), and it plays an essential role in promotion of inflammatory responses (Boshra et al., 2006). These responses in the NC group are probably linked to the ongoing inflammation in the kidney tissue caused by nephrocalcinosis. This was also evident by the increased expression of several immune cell markers such as MHC II and CD22. The lower expression of CD2 and other markers of the adaptive immune system suggests that the innate system was mainly involved. It is worth noting that the cases of nephrocalcinosis investigated in this paper involve rupture of the basal membrane of one or more nephrons, causing interaction between the mineral deposits and interstitial immune tissue. Further studies should investigate whether the same level of immune regulation can be seen in fish with mineral deposits retained within the excretory system without the involvement of interstitial tissue. Besides nephrocalcinosis, mineral deposits are known to form within various internal organs of salmonids such as the gastrointestinal wall (Roberts & Rodger, 2012), pseudobranch, choroid plexus of the eye,

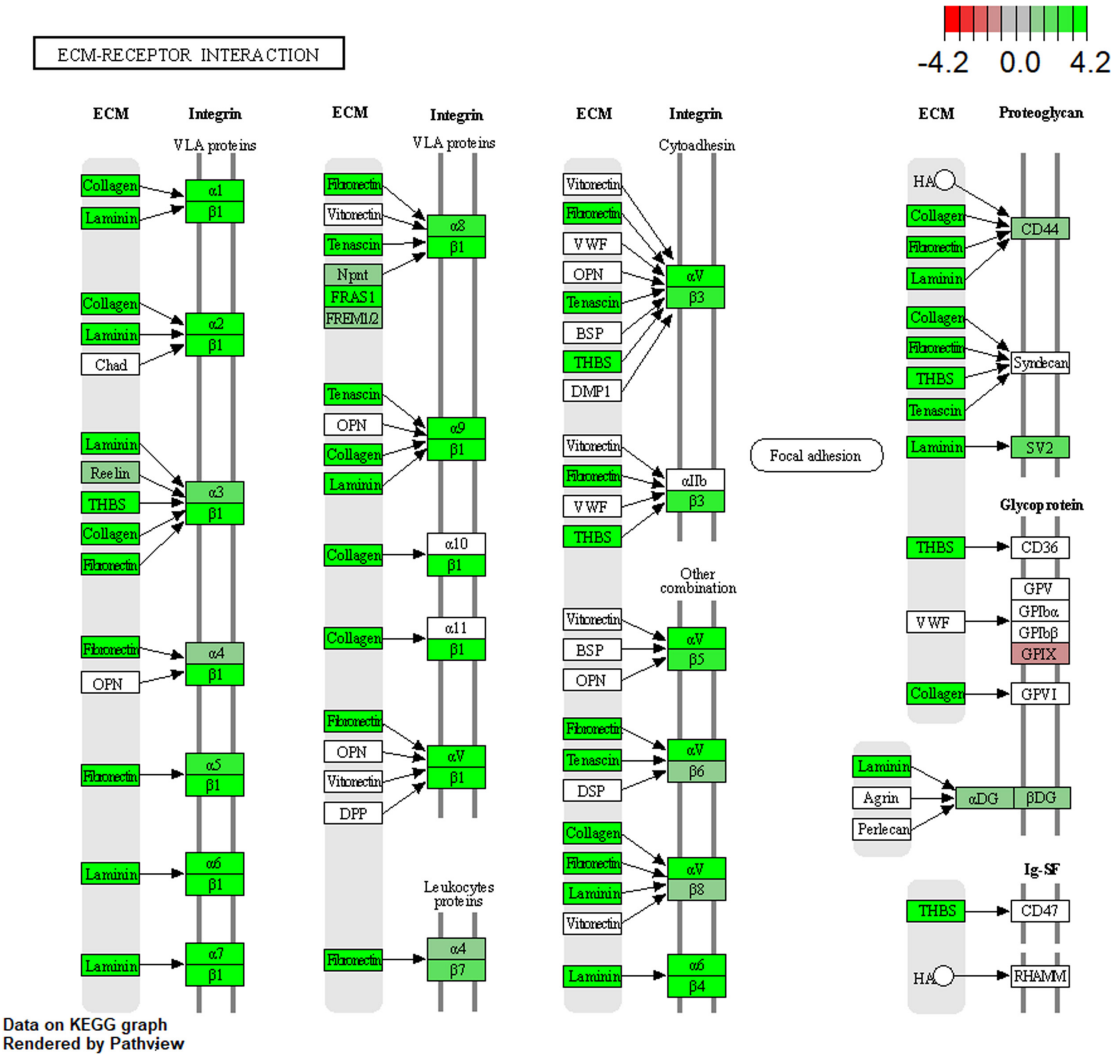

FIGURE 9 Pathway map sasa04512—ECM-receptor interaction. The protein EC number within each box is given in either green, red or white, indicating upregulation, downregulation or no change, respectively, with increased colour intensity indicating increased log2fold change

heart valves, compact layer or the cardiac ventricle, skeletal muscle and more often manifested as dystrophic calcification of parenchymatous cells in the resident tissue (Alf Dalum, personal observation). However, the causality of such soft-tissue mineralization, and a possible relation with nephrocalcinosis, has to our knowledge not been investigated. In our study, only renal tissue was subjected to histological investigation, and we cannot exclude a possible involvement of mineral deposits in other soft tissues.

Along with the inflammatory repair processes, transcriptomic data revealed significant upregulation of several repair and recruitment pathways such as membrane phospholipid reshuffling and synthesis (Kennedy pathway for DAG synthesis, PE, PC, PLA2, SCD-1 FATP1/4 and RXR), cell-to-cell communication, adherence and tight junctions, cytoskeletal functions and extracellular matrix (ECM), including many collagens, integrins, cadherins, laminins and thrombospondins essential for maintaining tissue structure and function (Webster et al., 2018). Upregulation of prostacyclin pathways can also be viewed as an attempt to inhibit blood clot at the damaged sites. The upregulation of some metabolic pathways (and claudin 11) appeared to be related to neuronal synthesis/maintenance (aspartyl-glutamate, citryl-glutamate and acetylcholine). It was interesting to note that in the cell adhesion pathway, many 'tight' sealing claudins like 1, 4, 5, 11 were upregulated while many 'leaky' pore-forming claudins (2, 10) were downregulated in NC fish, which could indicate

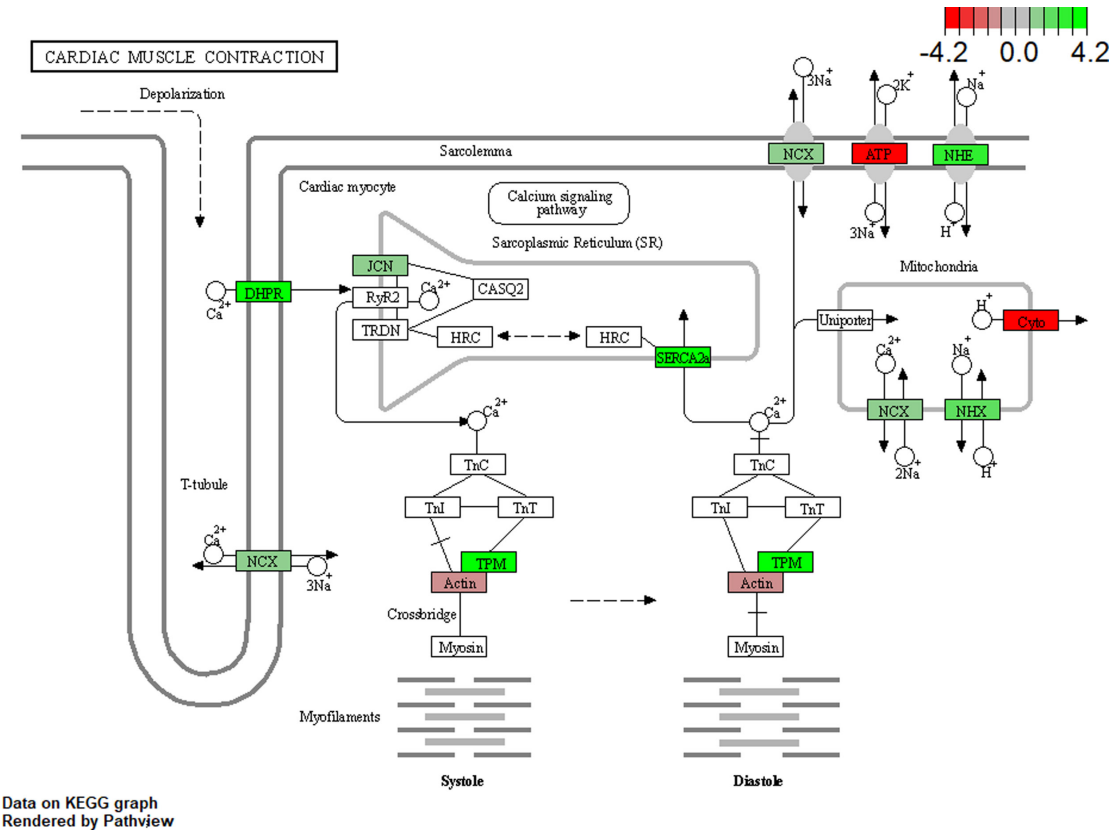

FIGURE 10 Pathway map sasa04260—cardiac muscle contraction. The protein EC number within each box is given in either green, red or white, indicating upregulation, downregulation or no change, respectively, with increased colour intensity indicating increased log2fold change

an attempt to seal the epithelial cell lining during nephrocalcinosis. In addition, Li et al. (2018) found that claudins may have vital functions in the immune responses in fish gill infections, which strengthens the observation that claudins are involved in immune responses in fish.

A notable observation in NC fish transcriptomics was the massive downregulation of most NADH/ATP energy-generating pathways (TCA-cycle and respiratory chain) including many pathways like amino acid metabolism and fatty acid β-oxidation that would normally feed carbon into the citric acid cycle. This suggests a malfunctional kidney with limited capacity for maintenance and repair. The increase in plasma AST and divalent ions, normally excreted over the kidney (magnesium, calcium and phosphate) (Beyenbach, 2000; Li et al., 2011; Nieves-Puigdoller et al., 2007; Singh et al., 2002; Wagner & Congleton, 2004), supports this notion. Another possible sign of kidney malfunction could be seen in the increased plasma lactate in NC fish. In clinical medicine, hyperlactatemia is indicative for kidney disease (Phypers & Pierce, 2006) and could be the consequence of impaired ATP production and perfusion. It is also interesting to note that Santis et al. (2015) found the same marked downregulation of metabolic pathways in their study of inflammation in the intestine caused by soybean protein. They also suggested that impaired metabolism could be a consequence of tissue malfunction.

5 | CONCLUSION

The transcriptome profiles of salmon affected by severe nephrocalcinosis revealed an upregulation of inflammation and repair processes together with a massive shut down of metabolism. These responses were most likely related to the severe tissue damage observed on histology resulting in kidney failure. The results of this study support that nephrocalcinosis is a major welfare challenge and most likely make the salmon more sensitive to additional stressors. The reduced renal function may have fatal consequences for the fish in the nurseries and should be further investigated.

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CONFLICT OF INTEREST

The authors declares that they have no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

All data are stored at Aqua Kompetanse AS according to internal guidelines and are available on request.

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SUPPORTING INFORMATION

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PAPER III

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SHORT COMMUNICATION

Journal of Fish Diseases WILEY

Radiological detection of nephrocalcinosis in farmed Atlantic salmon *Salmo salar* L.

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1 | INTRODUCTION

Nephrocalcinosis is a common disorder in nurseries in Norway (Klykken, Reed, et al., 2022) and was reported as one of the main welfare challenges in farmed salmon by The Norwegian Fish Health Report of 2019 (Sommerset et al., 2020).

Nephrocalcinosis is described as deposits of minerals within the kidneys (Bruno, 1996), that can disturb kidney function, which in turn can have dramatic consequences on fish performance and survival. The aetiology is most likely related to environmental factors, and suboptimal water quality has been indicated in several studies as the main risk factor (Fivelstad et al., 1999; Fivelstad et al., 2003; Khan et al., 2018; Fivelstad et al., 2015; Good et al., 2010; Lewisch et al., 2013; Chen et al., 2001). Newly conducted research suggested that osmoregulatory stress may be the trigger for nephrocalcinosis (Boissonnot et al., 2022).

Regardless of the severity of the condition, fish rarely present external signs, and it is thus challenging to monitor its prevalence and development. Present diagnostic methods require euthanasia as they consist of visually scoring the accumulation of deposits and the severity of lesions. Macroscopic assessments of necropsied fish are often imprecise, since small deposits are rarely visible to the naked eye, and histopathology is therefore considered as the best existing diagnostic method (Klykken, Boissonnot, et al., 2022). Research on, and monitoring of, nephrocalcinosis has been greatly hampered by the lack of non-invasive methods of assessing the presence and severity of this condition, as it is not possible to follow the development of the disease in single individuals, and as the number of sampled fish is limited due to ethical reasons.

Radiology has previously been used for assessing vertebrae deformities in Atlantic salmon (Drábiková et al., 2021; Holm et al., 2020), based on the classification scheme developed by Witten et al. (2009), and there has been a rapid development of the technology (Ou et al., 2021) including portable systems, which allow efficient in situ diagnosis. Nephrocalcinosis in Atlantic salmon is mainly identified as amorphous carbonate apatite, a calcium-dominated mineral (Klykken, Reed, et al., 2022), and it has previously been demonstrated that this mineral composition is suitable for x-ray detection (Smith & Lehr, 1966). Radiology is non-invasive and can be performed on anaesthetized fish, enabling assessment of nephrocalcinosis without euthanazing the fish. A non-invasive method for assessing nephrocalcinosis would allow for monitoring of the condition over time on an individual level and would be ethically and economically preferable. We have therefore explored radiology as a possible tool to detect and evaluate the severity of nephrocalcinosis, comparing it with histological scoring.

2 | MATERIAL AND METHODS

A total of 80 farmed Atlantic salmon were sampled from two recirculating aquaculture system facilities in Mid-Norway in April 2022. They were randomly sampled among visually healthy individuals (normal swimming behaviour, absence of external injuries/lesions and no sign of emaciation). The fish were not starved before sampling, and they were killed with an overdose of Benzoak VET (200–400 mg/L) followed by a sharp blow to the head according to Norwegian legislation (Akvakulturdriftsforskriften, 2008). Apart from nephrocalcinosis, no health issues were observed. Size measurements of individual fish included body weight in g (±1 g), fork length in cm (±0.5 cm), body thickness in cm (±0.1 cm) and condition factor: CF = $100 W/L^3$.

Left anterior lateral view radiographs were taken of smolts (average weight 198 g) using a standard portable x-ray unit (Econet meX+40; calibration: kV: 40, mAs: 5) with a digital plate measuring 25×32 cm and subsequently digitized using an VIVX-V2532P and VXvue. Distance between shutter opening and the fish was approximately 80 cm. The staff wore a lead apron and stood 2 m from the digital plate, resulting in negligible radiation exposure. After radiological imaging, the fish were dissected and eviscerated, and a picture was taken of the kidney tissues. Distribution and density of mineral deposits within the kidney was evaluated on radiographs in DICOM format in QXLink Portable Viewer (Vieworks, Republic of KOREA).

Whole kidney tissues were sampled from all individuals for histological analysis of nephrocalcinosis. The kidney tissues were fixed in 4% phosphate-buffered formaldehyde solution, embedded in paraffin wax and routinely processed (Suvarna et al., 2018). The kidney tissues were sectioned in the anterior–posterior direction in the para-sagittal plane so that the whole length of the kidney was evaluated for each fish. Due to space restrictions, segments of approximately 2.5 cm were made and oriented in a consecutive fashion from anterior to posterior on the resulting slide. All sections were stained by haematoxylin and eosin. The histopathological diagnosis of nephrocalcinosis was defined as the presence of amorphous, basophilic deposits in tubules, collecting ducts and excretory ducts. Histopathological nephrocalcinosis scoring was performed according to Klykken, Reed, et al. (2022).

Initially, histopathological nephrocalcinosis scoring was undertaken for all 80 fish. From this, a subsample of 17 fish was chosen to encompass all degrees of histological manifestation of nephrocalcinosis, and the radiographs of the corresponding fish were investigated. Based on those, we proposed five different categories for radiological scoring of nephrocalcinosis (herby referred to as radiological score) as given in Table 1 and Figure 1, with the sum of scores from each category making up the radiological score.

To validate the preliminary scoring system based on radiology, 63 fish were diagnosed on radiographic images only and then checked by comparing the results with the histological assessment. The validity of the preliminary model was assessed as the degree to which

TABLE 1 Categories for radiographic assessment of nephrocalcinosis

Parameter	Score	Assessment
Density of deposits	0	Not detected (Figure 1-0)
	1	Densities comparable to the spongiosa or less (Figure 1-1)
	2	Densities between densities comparable to spongiosa and the endplates of a random vertebrae (Figure 1-2)
	3	Densities comparable to the endplates of a random vertebrae (Figure 1-3)
Spatial distribution of mineral deposits	0	Not detected
	1	Only present in the collecting ducts
	2	Present in half of the space between the vertebrae column and the swim bladder (Figure 2-B2)
	3	Present in 75% of space between the vertebrae column and the swim bladder (Figure 2-C2)
	4	Present in nearly all kidney tissue
Presence of deposits in the total length of the kidney	0	Not detected
	1	<25% of the total length
	2	25%–50% of the total length (Figure 2-B3)
	3	50%–75% of the total length (Figure 2-C3)
	4	>75% of the total length (Figure 2-D3)
Presence in urinary bladder	0	Not detected
	1	Detected (Figure 2-B3)
Deviating ventral contour delimitation of the kidney	0	No deviation detected
	1	Deviation detected (see supplementary)

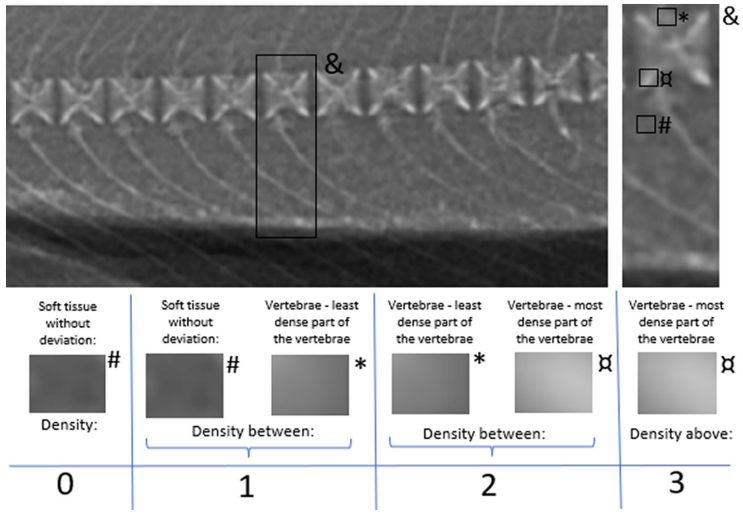

FIGURE 1 Density assessment of mineral deposits compared with density of soft tissue (#) and vertebral structures: spongiosa (*) and endplates (¤).

scores based on radiology were correlated with the histology score, based on the assumption that histology scoring was sufficiently objective. Correlation was checked with Pearson correlation test.

3 | RESULTS AND DISCUSSION

The distribution and density of mineral deposits were clearly observed on the x-ray images (Figure 2), which offers promising prospects for the development of a non-invasive assessment of nephrocalcinosis in farmed salmon.

The sensitivity of radiology for nephrocalcinosis was high, as 93.2% of the fish (n = 55) histologically diagnosed with nephrocalcinosis were also diagnosed with radiology (Table 2). The remaining 6.8% (n = 4) that were not detected with radiology were all diagnosed with mild changes with histology. On the opposite, three individuals were diagnosed with nephrocalcinosis with radiology, but not detected with histology. These were assessed with sparse amounts of mineral deposits. The correlation between the total histology score and the radiological assessment (Figure 3) was significant but low (Yadav, 2018). One possible explanation for these inconsistencies could be that mild changes, according to the histological scoring model, usually refer to sparse amounts of mineral deposits in collecting ducts and tubules (Klykken, Reed, et al., 2022). It is, therefore, likely that the density or the proportion of mineral deposits were too small to be detected by radiography. We also observed background artefacts on some of the radiographs that may have affected the radiological assessment of nephrocalcinosis. To increase the specificity of radiology, background artefacts should be minimized. For example, breaching the scale pockets could lead to disturbances with several scales ending on top of each other in an unnatural fashion, causing disturbances to the radiographic image. Background artefacts may also have contributed to the radiological detection of the three fishes, which were considered healthy with histology (Table 2).

Another possible explanation for these inconsistencies is related to the limitations of histopathology itself. The histopathological nephrocalcinosis score is a semi-quantitative scoring model based on changes in the kidney according to the known principles of pathology. Histopathological changes are divided into four subcategories (presence of deposits, epithelial degeneration and/or necrosis, pathological changes in the glomeruli and pathological changes in the interstitial tissue; Klykken, Reed, et al., 2022). The histological section represents a two-dimensional thin slide (approximately 3 μm) of a relatively large organ, and there might be deposits in a plane that is not included in the section. It is, therefore, possible that we were able to detect deposits with radiology that we failed to observe with histopathology. This should be further investigated as it is not possible to conclude based on our study.

It is also possible that the low correlation could be explained by the fact that radiology can only detect the first category in the histopathologic scoring model, that is, the presence of deposits. Fish with severe changes in the kidney tended to have larger spatial distribution of minerals on radiography. In addition, whether we observed mineral deposits in the urinary bladder was not correlated with the overall histological score, and the radiological categories should be further evaluated.

The use of portable radiography is advantageous as it allows for a fast and cost-effective diagnosis of mineral deposits characteristic for nephrocalcinosis that can be performed on a large number of sedated individuals at each facility. The Norwegian legislation requires, however, that the person conducting radiography (operator) receives a short training and use radiation protection, as radiography consists of x-ray radiation, which can potentially pose a risk for the operator (Zamanian & Hardiman, 2005). The voltage, shutter speed and exposure time settings used in this study resulted in radiation doses of 0.001 mSv/h measured at a distance of 20 cm from the digital plate. In this study, the operator was wearing a lead apron, standing 2 m from the digital plate,

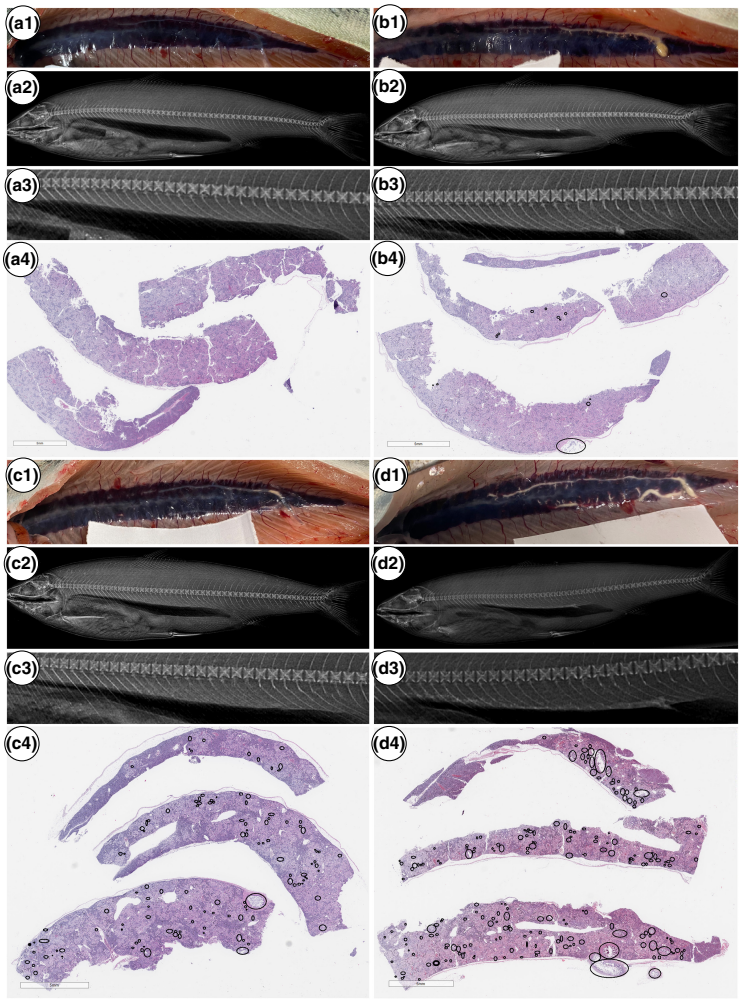

FIGURE 2 Examples of macroscopical presentation (1), radiological imaging (2 (whole fish) and 3 (focus on kidney region)) and histology (4 (section of the whole kidney from anterior to posterior part; regions with changes associated with nephrocalcinosis indicated by black ellipses), from normal fish (a) and fish with different grading of nephrocalcinosis ranging from mild (b), moderate (c) to severe (d) as evaluated from radiological and histological evaluation.

TABLE 2 Number of fish with nephrocalcinosis detected on radiology and histology

	Radiology positive	Radiology negative	Total
Histology positive	55	4	59
Histology negative	3	1	4
Total	58	5	63

resulting in effective dose of 0.00002 μSv, which is quite negligible. For comparison, will a single domestic flight in the US give an effective dose of 3-6 μSv (United Nations Scientific Committee on the Effects of Atomic Energy, 2010).

Regarding the future use of radiology to assess nephrocalcinosis, one should be aware of the limitations of the method, since soft tissue changes not including mineralization cannot be seen on radiographs. In general, it is not possible to evaluate the degree of soft tissue lesions associated with deposits based on radiology alone. Another major limitation is the fact that the radiology cannot differentiate between different types of pathologies associated with mineral deposits in kidney tissue, meaning that conditions such as chronic granulomatous inflammation with secondary central calcification cannot be differentiated from deposits due to nephrocalcinosis. Histopathology will, therefore, still be crucial for the evaluation of soft tissue changes and for definitive confirmation of diagnosis.

The method should be further developed to establish cut-off categories, similar to those of the histological model. To do so, a larger sample size should be assessed, also including several individuals without nephrocalcinosis as 'control', to evaluate the proportion of false and true negatives. We believe that this will increase the sensitivity and specificity of radiology.

In conclusion, we are confident that radiology is a suitable diagnostic tool for assessing mineral deposits (amount and distributions) associated with nephrocalcinosis in farmed Atlantic salmon, with minimal risk to the operator when appropriate measures are taken. The methodology is significantly more cost-effective than histology

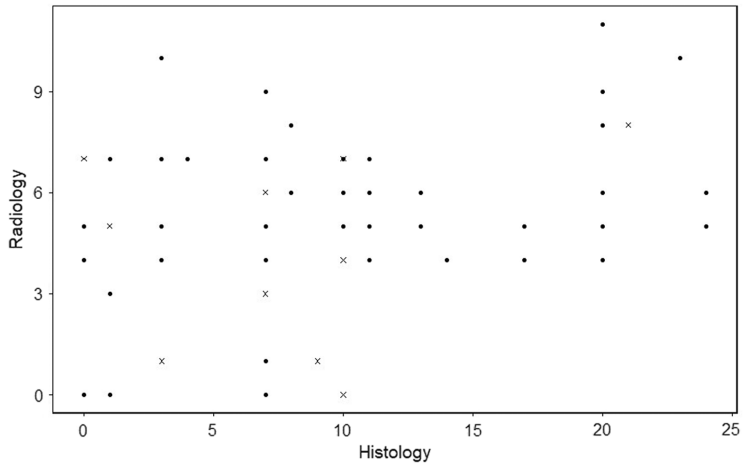

FIGURE 3 Scatterplot of radiological (*y*-axis) assessment and histological assessment (*x*-axis). Each point represents a single individual. Those marked as 'x' indicate radiographs with background disturbances. *df* = 61, *p*-value = .0084, *r*-square = 0.33.

with faster response time and reduces drastically the number of fish that need to be euthanized.

We recommend further developing radiology for the assessment of nephrocalcinosis in fish. This non-lethal method will make it possible to assess the disease progression on individual and group level over time, as well as exploring modes of prevention and treatments, greatly increasing the possibility of discovering its aetiology.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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PAPER IV

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RESEARCH ARTICLE

Journal of Fish Diseases WILEY

Nephrocalcinosis in juvenile farmed Atlantic Salmon (*Salmo salar*) may be linked to osmoregulatory stress

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Abstract

Nephrocalcinosis is a widespread challenge in intensive production of salmon smolt. There is however no consensus on its aetiology, which makes it problematic to implement proper measures to limit its development. We performed a survey of nephrocalcinosis prevalence and environmental factors in 11 different hatcheries in Mid-Norway as well as a 6-month monitoring in one of the hatcheries. A multivariate analysis indicated that the most influencing factor for the prevalence of nephrocalcinosis was the supplementation of sea water during smolt production. In the 6-month monitoring, the hatchery introduced salinity in the production water prior to the change in day length. Mismatch in those environmental signals may increase the risk for developing nephrocalcinosis. Salinity fluctuations prior to smoltification can cause osmotic stress and result in unbalanced levels of ions in fish blood. This was clearly illustrated in our study, as the fish experienced chronic hypercalcaemia and hypermagnesaemia. Both magnesium and calcium are excreted over the kidneys and it is possible that their prolonged, elevated levels in plasma resulted in an oversaturation of the urine when finally excreted. This again could have led to the aggregation of calcium deposits within the kidney. This study indicates a relationship between osmotic stress induced by salinity changes in juvenile Atlantic salmon and the development of nephrocalcinosis. Other factors that may affect the severity of nephrocalcinosis are currently subjects for discussion.

KEYWORDS
Atlantic salmon, hypercalcaemia, nephrocalcinosis, osmoregulatory stress, salinity

1 | INTRODUCTION

The salmon industry in Norway contributes to a yearly landing value of 8.6 billion EUR (Fiskeridirektoratet, 2021) and is one of the most important industries in rural Norway (Olaussen, 2018). In 2021, 412 million smolts were sold to sea facilities (Fiskeridirektoratet, 2021) with an increasing demand for transferring fish all year round (Tang et al., 2022).

In commercial hatcheries, salmon are normally raised in constant light (LD24:0) from first feeding until smoltification is induced by introducing an artificial winter signal (light during 12 h, followed by darkness for 12 h; LD12:12) followed by LD24:0 (Martinez et al., 2021). The change in photoperiod induces an increase in expression of Na^+, K^+-ATPase α1a isoform and a reduction in expression of the α1b isoform (Nilsen et al., 2007) in the gills,

reflecting the development of hypo-osmoregulatory ability and seawater tolerance necessary for successful smoltification (Handeland et al., 2003; McCormick et al., 1987; Stefansson et al., 1991). When the hypo-osmoregulatory capacity of the salmon is sufficiently high (Mortensen & Damsgård, 1998), the smolts are considered to be in the 'smolt-window', a relatively short period when they can be successfully transferred from freshwater to sea water (Lundqvist & Eriksson, 1985).

The Norwegian Veterinary Institute reports nephrocalcinosis as an increasing welfare issue in Norwegian smolt production (Jansen et al., 2021). Nephrocalcinosis is characterized by calcium deposits in the kidney tubules and collecting ducts (Bruno, 1996). In Atlantic salmon, the mineral deposits, characteristic for nephrocalcinosis, mainly consist of amorphous carbonate apatite (amCAP; Klykken et al., 2022), which is made up mainly of calcium and phosphate. Calcium homeostasis in teleosts is regulated by several hormones, for example stanniocalcin (STC), calcitonin (CT) and calmodulin (CAM; Chan, 1972). STC is one of the primary hypocalcaemic hormones in fish and is present as two isoforms, STC1 and STC2 that have been detected in renal tissue (Amemiya et al., 2002; McCudden et al., 2001). STC1 exerts an inhibitory effect on Ca^{2+} uptake in the gill by negatively regulating gene expression of epithelial channels (ECaC; Tseng et al., 2009). It also decreases intestinal uptake of calcium (Sundell et al., 1992) and stimulates the reabsorption of phosphate in the kidney (Lu et al., 1994). STC2 acts by inhibiting the uptake of Ca^{2+} in the gills and intestine (Domínguez et al., 2019). CT exerts its hypocalcaemic action by specifically stimulating osteoblast activity to form bones and scales (Domínguez et al., 2019). CAM is one of the most abundant Ca^{2+} sensor proteins in vertebrates (Ikura & Ames, 2006), which protects against calcium toxicity by keeping the intracellular concentration of calcium low (Wongdee & Charoenphandhu, 2013). CAM has an extracellular role in regulating the permeability of fish skin to water and ions (Flik et al., 1984) and has a high affinity for calcium (Lo et al., 1994). Shavit et al. (2015) suggested that local dysregulation of calcium homeostasis in the renal interstitium may play a key role in the pathogenesis of nephrocalcinosis in humans, but this has not been investigated in fish yet.

There is to date no consensus on the aetiology of nephrocalcinosis. Several studies conducted on farmed Atlantic salmon have indicated that both acute and long-term exposure to high levels of CO_2 in the ambient water can cause nephrocalcinosis (Fivelstad et al., 1999, 2003, 2018; Khan et al., 2018). Similar results have been observed in studies with rainbow trout (*Oncorhynchus mykiss*; Smart et al., 1979; Good et al., 2010), Atlantic cod (*Gadus morhua*; Damsgård et al., 2011) and spotted wolfish (*Anarhichas minor*; Foss et al., 2003). In contrast, other comparable studies on elevated levels of CO_2 did not find any signs of development of nephrocalcinosis in Atlantic salmon (Fivelstad et al., 1998, 2015; Good et al., 2010, 2018; Mota et al., 2019). This may indicate that other environmental factors rather than, or in addition to suboptimal water CO_2 levels, must play an important role in the development of nephrocalcinosis. High levels of phosphate combined with high pH in the water have been suggested to cause nephrocalcinosis (Lewisch et al., 2013). For Nile tilapia (*Oreochromis niloticus*) reared in RAS, the prevalence of nephrocalcinosis was reduced by changing the source of alkalinity from $CaCO_3$ to $NaHCO_3$ (Chen et al., 2001).

To contribute to the common effort to understand the aetiology of nephrocalcinosis in Atlantic salmon, we have conducted a survey of nephrocalcinosis in 11 different hatcheries and a broad selection of water quality and feed composition parameters were analysed. Based on the results from this survey, we performed a long-term monitoring of the development of nephrocalcinosis in one of the hatcheries that had a high prevalence (of nephrocalcinosis). We also monitored production conditions that may have increased the risk of developing nephrocalcinosis (temperature, CO_2, O_2, pH, salinity and L:D).

2 | MATERIALS AND METHODS

2.1 | Fish sampling

A survey was conducted with a total of 360 farmed Atlantic salmon that were sampled from 12 fish groups in 11 different hatcheries, both flow through (FT) and recirculation aquaculture systems (RAS) in Mid-Norway from October 2019 to June 2020 (please see Klykken et al. (2022) for more details). No fish were exposed to experimental manipulation, and the sample material therefore represents fish under conventional farming conditions. A total of 30 fish from each facility were randomly sampled among visually healthy individuals within 2 weeks prior to sea transfer (Mean weight: 160.5 ± 85.1 g). The fish were not starved before sampling and were killed with an overdose of Benazok VET (200–400 mg/L) followed by a sharp blow to the head according to Norwegian legislation (Akvakulturdriftsforskriften, 2008). A general health assessment and an evaluation of morphological changes related to parr–smolt transformation (smoltification; silver colour, darker fin and parr marks) were performed at each sampling.

A long-term monitoring of nephrocalcinosis was performed at one of the flow-through facilities (8). The facility was selected based on having a historically high prevalence of nephrocalcinosis and a production protocol that included the use of sea water supplementation. Sampling was performed monthly from two tanks, from November 2020 (mean weight: 110.6 ± 14.1 g) to April 2021 (mean weight: 163.4 ± 33.6 g). Fish were randomly sampled (November: $n = 30$, December–February: $n = 60$, March: $n = 52$, April: $n = 51$), using the same protocol as during the survey. Seventeen fish were diagnosed with haemorrhagic smolt syndrome (HSS) and were excluded from the dataset as they could create bias in the analyses.

2.2 | Histopathology

Tissues from mid-kidney were sampled from all individuals for histopathological analysis of nephrocalcinosis. The kidney tissues were fixed in 4% formaldehyde solution, embedded in paraffin wax and routinely processed (Suvarna et al., 2019). All sections were stained by

haematoxylin and eosin, and a selection of sections was stained with von Kossa stain (Rungby et al., 1993). The histopathological diagnosis of nephrocalcinosis was undertaken according to Klykken et al. (2022).

2.3 | Blood chemistry

Blood samples were taken from all individuals in the long-term monitoring of nephrocalcinosis. Vacutainer tubes (Becton-Dickinson) with lithium heparin as anticoagulant were used to collect blood from the caudal vein immediately after euthanasia. After thorough mixing, the samples were centrifuged at 13,500 rpm for 5 min (VWR Mikrostar 12, 12 × 1.5/2.0 mL) and the plasma was transferred to Eppendorf tubes and kept frozen (minimum −20°C) until analysis.

The following parameters were measured at Aqua Kompetanse's laboratory using an automated dry chemistry analyser (CatalystOne, IDEXX Laboratories, Westbrook, ME, (Boes et al., 2018)): aspartate aminotransferase (AST), calcium (Ca), sodium (Na), potassium (K), chloride (Cl) and magnesium (Mg). Each of the assays used a standard kit developed for the automated analyser (CatalystOne, IDEXX Laboratories, Westbrook, ME). Relevant blood chemistry parameters were chosen based on Klykken et al. (2022).

2.4 | Water quality

Husbandry information was collected from the different hatcheries of the survey, and additional triplicate water samples from the production water were sampled simultaneously with the fish sampling. 0.5-L plastic bottles were used for sampling and immediately stored in ice boxes and kept at 4°C until analysis. The photometer WTW Photoflex Turb Set (WTW) was used to measure concentration of calcium, magnesium, phosphate, ammonium, potassium, sodium, nitrate, nitrite, sulphate and iron. Alkalinity was indirectly measured with a pH meter and the Orion Total Alkalinity test kit (Orion Research, Inc.).

In the long-term monitoring, the water quality was routinely monitored by the facility and kept within recommended ranges for Atlantic salmon (Noble et al., 2018). In addition, triplicate water samples from each tank were collected monthly (same dates as fish sampling) in 0.5-L plastic bottles and immediately stored in ice boxes and kept at 4°C until analysis. The photometer WTW Photoflex Turb Set (WTW) was used to measure concentration of calcium, magnesium, phosphate and iron. Alkalinity was indirectly measured with a pH meter and the Orion Total Alkalinity test kit (Orion Research, Inc.).

2.5 | Feed composition

Feed samples were collected directly from the automatic feeder of the fish tanks from the hatcheries in the survey. The samples were kept frozen (minimum −20°C) until analysis. The analyses were performed by the accredited laboratory Sintef Norlab AS according to standards NMKL23 (gravimetric determination), NMKL6 (Kjeldahl-N), ICP-MS and internal methods.

2.6 | Gene expression by qPCR

2.6.1 | Kidney gene expression of calcium-related genes

In the long-term study, mid-kidney tissue was isolated and immediately transferred into tubes containing RNAlater© (Life technologies) and stored at 4°C for 24 h before storage at −20°C until analysis. Direct-zol™ RNA extraction kit (Zymo research corporation) was used for extracting total RNA from frozen kidney tissues, followed by RNA integrity check and quantification using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Reverse transcriptase (quantitative) polymerase chain reaction (RT-qPCR) was performed to quantify changes in the expression of CT, CAM, STC and MGP in the kidney. Complementary DNA (cDNA) synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad). Followed by the preparation of qPCR reaction mix (20 µL) containing—5 µL of diluted cDNA, 0.5 µM each of the forward and reverse primers, 1× iTaq SYBR green supermix with ROX (Bio-Rad Laboratories) and amplified using Mx3000P real-time PCR machine (Stratagene). Detailed procedure for the quantification of transcript expression is presented in Appendix S1. Primer pair sequences used for transcript amplification are shown in Table S1. The normalized expression was calculated using the relative expression ratio method described by Pfaffl (2001).

2.6.2 | Gill and kidney Na^+, K^+ ATPase (NKA) gene expression

In the long-term survey, gill and kidney tissue were isolated and immediately transferred into tubes containing RNAlater© (Life technologies) and stored at 4°C for 24 h before storage at −20°C until analysis. Measurement of gill and kidney ATPase expression was performed by Pharmaq Analytiq AS. Their product 'Smoltvision' was used, measuring the expression of three different gene markers: α1a NKA isoform, α1b NKA isoform and the Na^+, K^+, $2Cl^-$ (NKCC1a) cotransporter. The normalized expression was calculated using the relative expression ratio method described by Pfaffl (2001).

2.7 | Statistical analysis

All statistical analyses were performed using R software 4.1.0 (Team, 2017). An exploratory data analysis (EDA) with principal component analysis (PCA) and partial least squares (PLS) was used to explore the possible relationship between different environmental

factors, biological parameters and the prevalence of nephrocalcinosis in 12 different fish groups. The predictor variables included the following parameters: biological measurements (plasma ALKP, plasma ALT, plasma AST, gill ATPase ratio, plasma Ca, plasma CK, plasma Cl, plasma K, plasma Mg, plasma Na, plasma Na/K ratio, plasma PO_4, weight, length, hearth deformities, light liver, swollen spleen, internal adhesions, cataract, fin injuries, gill deformities, jaw/snout wounds, melanin, scale loss and smolt characteristics); feed components (ash, calcium, carbohydrate, chloride, dry matter, fat, iron, potassium, magnesium, sodium, phosphorus, protein, vitamin D3 and water); and water quality measurements (alkalinity, ammonium, calcium, CO_2, iron, potassium, magnesium, sodium, nitrate, nitrite, O_2, pH, phosphate, sulphate, temperature, total nitrogen and turbidity). Rearing conditions also included: stocking density, production technology (FT/RAS) and pH buffer. See Appendix S2 and Appendix S3 for values.

PCA was performed on the full dataset, where the biological and environmental factors were used as explanatory variables, while the prevalence of nephrocalcinosis was plotted as a supplementary variable. The average contribution to the first five principal components was used as cut-off and only variables with contributions greater than this limit were considered important for contributing to the components, while the variables below were not included in the PLS regression. The number of components in the PLS regression model was chosen by using leave-one-out cross-validation, where the number of components which gave lowest root mean squared error (RMSE) was chosen.

Statistical analyses were performed on plasma chemistry and gene expression. Because of heavily skewed gene expression data, the gene expression data were log-transformed with base 2 before the analysis. Normality was tested with Shapiro–Wilks test. Because of non-normality, the Wilcoxon rank-sum test was used for group comparisons between healthy fish and fish with nephrocalcinosis. Comparison between sampling days and tanks was also performed with pairwise Wilcoxon rank-sum test. *p*-values were adjusted using Bonferroni correction. *p*-values ≤.05 were stated as significant. To measure the strength and direction of the monotonic relationship between prevalence of nephrocalcinosis and salinity, Spearman's rank correlation was used. All the results from the statistical analysis are provided in the supplementary material (Tables S7–S19 in supplementary).

3 | RESULTS

3.1 | Survey of 12 fish groups

3.1.1 | Prevalence before transfer to sea

Nephrocalcinosis was observed in all the fish groups in the survey, but the prevalence varied greatly between the hatcheries (Figure 1, see Klykken et al. (2022) for more details on the histology results). More than half of the individuals with nephrocalcinosis had mild changes in the kidney tissues (68%, Figure 1). The total proportion of fish with nephrocalcinosis was 45% in the surveyed hatcheries.

3.1.2 | Nephrocalcinosis in relation to external factors

The PLS regression model indicated that the supplementation of sea water in the production water and sulphate concentration were the two investigated parameters that explained the prevalence of nephrocalcinosis most (Figure 2). The model explained 74% of the variation in the response variable.

All hatcheries that had less than 25% nephrocalcinosis did not add sea water to their production water. On the opposite, all but one hatchery that had over 25% nephrocalcinosis added sea water in different concentrations (Figure 3). There was a significant positive

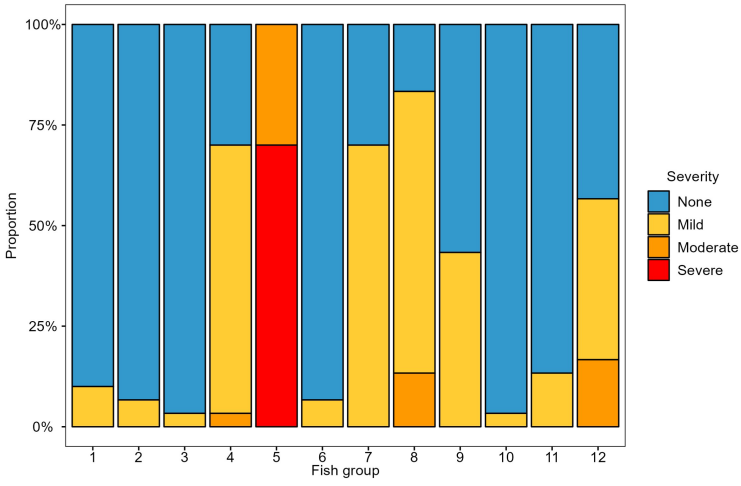

FIGURE 1 Severity of nephrocalcinosis in Atlantic salmon from different fish groups in Mid-Norway (Each group: n = 30). Yellow colour indicates mild changes, orange shows moderate changes, and red indicates severe changes in the kidney tissue as found from histopathological examination (Klykken et al., 2022).

correlation between the proportion of fish with nephrocalcinosis and the salinity in the hatchery (Spearman's rank correlation: $\rho = .89$, $p < .05$).

3.2 | Long-term survey in one hatchery

3.2.1 | Development of nephrocalcinosis

In the facility that was monitored over several months (November 2020–April 2021), there was an increase in both prevalence and severity of nephrocalcinosis in both tanks during the study period (Figure 4). Compared with the first 3 months (November, December and January), the prevalence was significantly higher than the other months of the study ($p < .05$). There was a clear increasing trend in the prevalence of nephrocalcinosis in fish from February until the end of the study (Figure 4).

3.2.2 | Environmental conditions

The fish were exposed to continuous light (LD24:0) from start-feeding until they reached a size of 30–40 g (September 2020). In early September, the light regime was switched to LD16:8 for 2 weeks and from late September to the end of January the fish were exposed to a 'winter signal' characterized by LD12:12 (Folkedal et al., 2010). From February and until transfer to sea, the fish were exposed to continuous light (LD24:0; Table. 1). The temperature decreased from 6°C in November to 3°C in April, while the O_2 saturation was kept at 85% (measured in the outlet) and the CO_2 below 6.0 mg/L during the whole sampling period. Salinity increased gradually over time with one increase in December and another in January (Figure 4). pH was not continuously logged by the facility, but was sporadically measured, and ranged between 6.6 and 7.0 for the sampling period. Alkalinity increased from November to February and then decrease in March and April. Ca^{2+} and Mg^{2+} concentrations increased from November to March (Table. 1), with a significantly elevated concentration in February, March and April compared with November and December.

3.2.3 | Blood chemistry

Since the blood chemistry did not significantly differ between fish with and without nephrocalcinosis and only for single time points for some of the parameters between the two tanks (Table S8 and Table S9), data from fish from the different tanks and groups were gathered for further analysis resulting in an increased sample size.

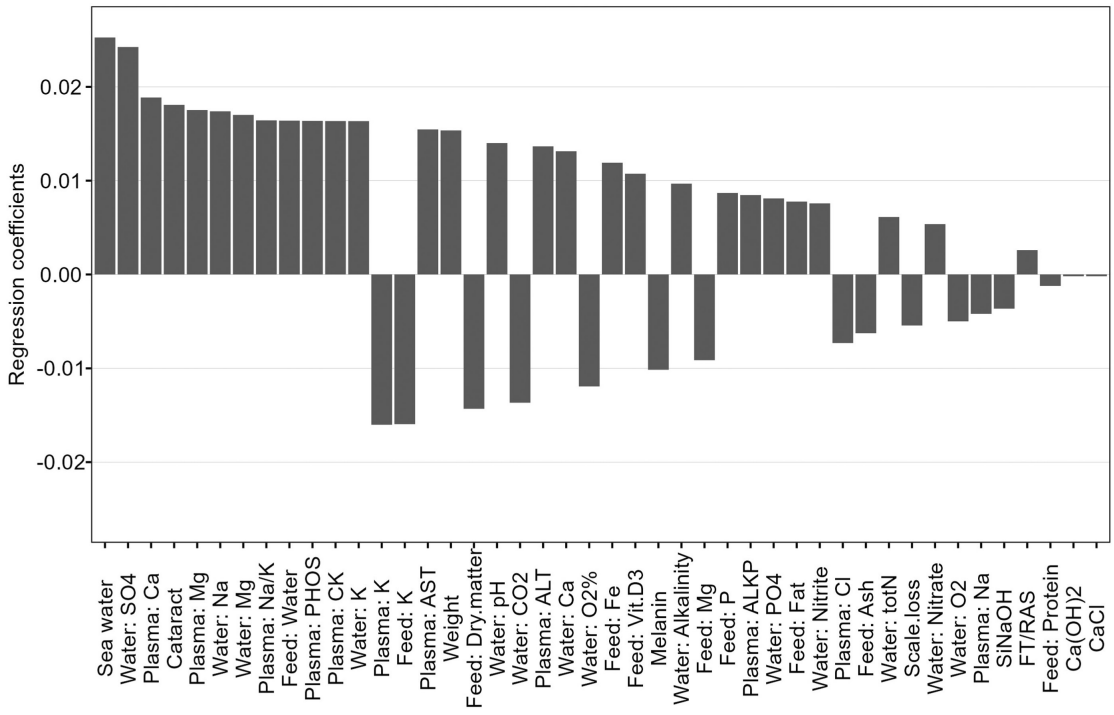

FIGURE 2 Regression coefficients from the partial least squares regression analysis of nephrocalcinosis with the covariates that were considered to have important contributing to the principal components in the full PCA (Appendix S4). Root mean square error (RMSE): 0.34.

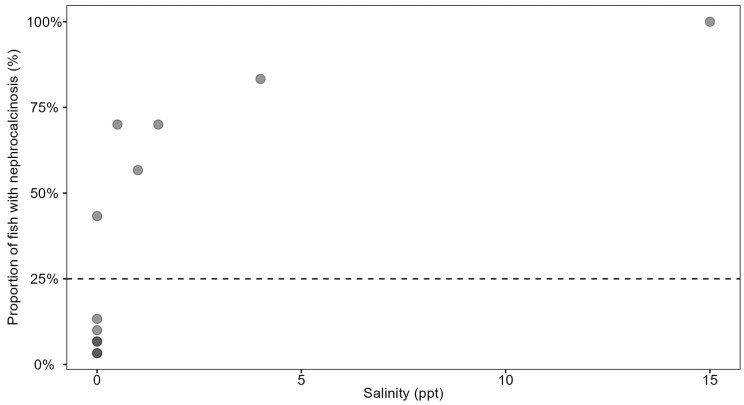

FIGURE 3 Proportion of fish affected by nephrocalcinosis as a function of salinity. Each point ($n = 30$) represents one fish group. Line indicating 25% prevalence of nephrocalcinosis.

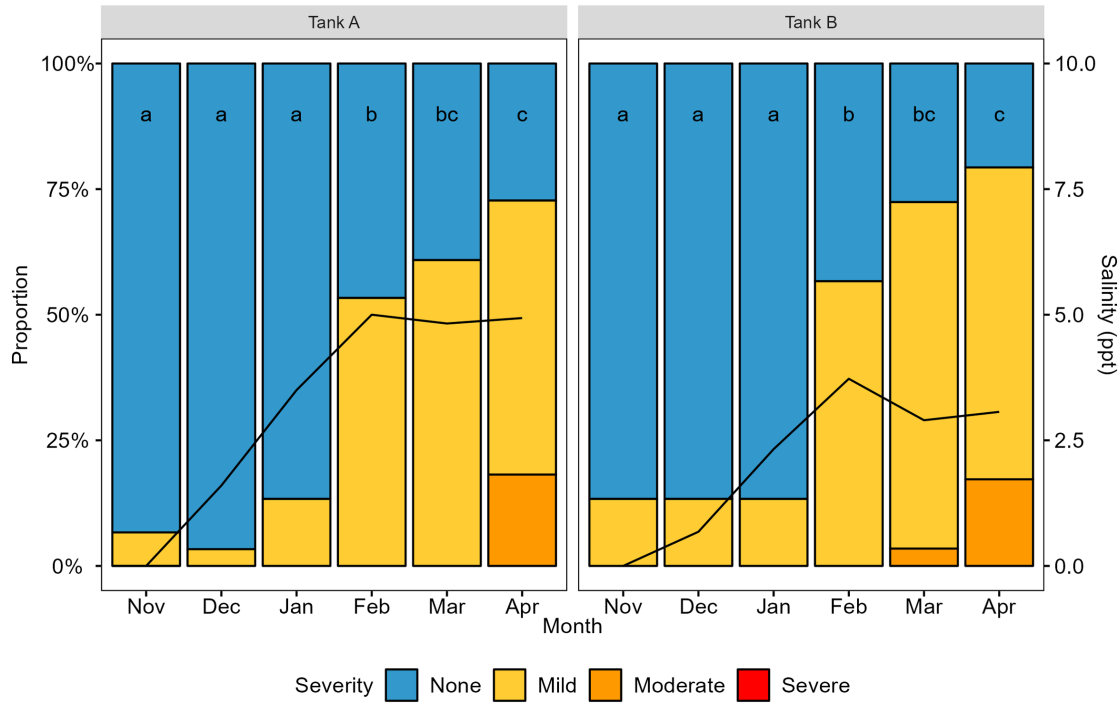

FIGURE 4 Prevalence and severity of nephrocalcinosis at different sampling points for tank A and B including salinity. Significant differences in nephrocalcinosis prevalence between sampling months are denoted with different letters ($p < .05$). Fish from tank A: (Nov; $n = 15$, Dec-Feb; $n = 30$, Mar; $n = 23$, Apr; $n = 22$. Fish from tank B: Nov; $n = 15$, Dec-Feb; $n = 30$, Mar; $n = 29$, Apr; $n = 29$). Black line shows salinity changes over time in tank A and B (ppt).

Most blood chemistry parameters measured changed during the survey period (Figure 5). The plasma concentrations of calcium and magnesium were significantly elevated in December and January compared with all other sampling points. There was a significant decrease in the concentration of AST from December to February and the chloride concentrations increased over time, while sodium did not show clear temporal patterns.

3.2.4 | Kidney gene expression

Fluid and electrolyte homeostasis

The kidney gene expression of NKA α-1a, NKA α-1b and NKCC1a did not significantly differ between fish with and without nephrocalcinosis and only for a single time point for one of the genes between the two tanks (Table S11 and Table S12), and data from fish from

TABLE 1 Environmental parameters for tank A and B from November 2020 to April 2021. Temperature (Temp., °C), oxygen saturation (O_2, %), light:darkness (L:D), CO_2 (mg/L), salinity (Sal, ppt), alkalinity (Alk, ppm $CaCO_3$), calcium (Ca, mg/L), magnesium (Mg, mg/L) and phosphate (PO_4, mg/L). Significant differences between months are indicated with different letters ($p > .05$).

	Nov	Dec	Jan	Feb	Mar	Apr
Temp.	6.0 ± 0.5	4.3 ± 0.5	3.4 ± 0.1	3.3 ± 0.1	3.2 ± 0.1	3.0 ± 0.2
O_2	85%	85%	85%	85%	85%	85%
L:D	12:12	12:12	12:12	24:0	24:0	24:0
Tank A						
CO_2	5.0	NA	4.0	4.0	2.0	NA
Sal	0 ± 0	1.6 ± 0.8	3.5 ± 1.1	5.0 ± 0.1	4.8 ± 0.2	4.9 ± 0.1
Alk	0.0 ± 0.0^{a}	11.2 ± 3.4^{ab}	16.0 ± 1.3^{b}	15.7 ± 0.4^{b}	15.3 ± 0.5^{b}	14.6 ± 0.6^{b}
Ca	12.0 ± 3.5^{a}	27.0 ± 4.4^{a}	56.0 ± 2.0^{b}	64.0 ± 3.0^{bc}	69.3 ± 0.6^{c}	70.1 ± 0.6^{c}
Mg	$<5.0^{a}$	59.7 ± 16.3^{a}	193.7 ± 50.6^{ab}	254.7 ± 2.1^{b}	161.0 ± 15.7^{b}	189.7 ± 11.2^{b}
PO_4	0.05 ± 0.04	0.1 ± 0.02	0.07 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
Tank B						
CO_2	5.0	NA	5.0	6.0	4.0	NA
Sal	0 ± 0	0.7 ± 0.3	2.3 ± 1.4	3.7 ± 0.0	2.9 ± 0.1	3.1 ± 0.0
Alk	2.2 ± 0.7^{a}	6.7 ± 1.6^{ab}	12.9 ± 0.5^{b}	13.4 ± 1.4^{b}	10.0 ± 1.2^{b}	9.6 ± 0.8^{b}
Ca	$<10^{a}$	15.0 ± 2.7^{a}	50.7 ± 6.7^{ab}	67.7 ± 13.6^{ab}	45.7 ± 2.1^{b}	44.3 ± 1.5^{b}
Mg	$<5^{a}$	48.4 ± 0.6^{b}	135.0 ± 4.6^{c}	226.7 ± 29.4^{cbd}	89.7 ± 4.7^{d}	91.7 ± 16.2^{abcd}
PO_4	0.04 ± 0.02^{ab}	0.2 ± 0.04^{ab}	0.09 ± 0.01^{a}	0.09 ± 0.02^{ab}	0.04 ± 0.01^{b}	0.06 ± 0.01^{b}

Note: Iron was below the limit of detection (LOD) (<0.05 mg/L) at all sampling times. a, b, c, d indicates significance levels of $p > .05$.

the different tanks and groups were therefore gathered for further analysis resulting in an increased sample size.

The results showed that gene expression of NKA α-1a and NKCC1a changed over time in the kidney tissue with a significant increase in March compared with earlier months (Figure 6.), while only small changes, without an apparent pattern, were observed for the NKA α-1b isoform.

Calcium signalling in the kidney

The kidney gene expression of calcium signalling-related genes did not significantly differ between fish with and without nephrocalcinosis with one exception (MGP in December), and only for single time points for one of the genes between the two tanks (CT, Table S14 and Table S15), data from fish from the different tanks and groups were therefore gathered for further analysis resulting in an increased sample size.

There were some variations over time in gene expression of calcium signalling-related genes (Figure 7). STC decreased from November until January and then increased with highest relative gene expression in April.

3.2.5 | Gill gene expression

The gill gene expression of NKA α-1a, NKA α-1b and NKCC1a did not significantly differ between fish with and without nephrocalcinosis, and only for single time points for the genes between the two tanks (Table S17 and Table S18), data from fish from the different tanks and groups were therefore gathered for further analysis resulting in an increased sample size.

The relative mRNA expression of the NKA α-1a isoform decreased from November to April (Figure 8). The relative expression of NKA α-1b isoform varied and showed a less distinct pattern (Figure 8). The Na+-K+-2Cl− (NKCC1a) cotransporter was within the recommended 20% of total gene expression at every sampling point (Figure 8).

4 | DISCUSSION

Previous studies that investigated the aetiology of nephrocalcinosis have pointed out seemingly contradictory explanatory factors, ranging from exposure to high CO_2 concentration in the water (Damsgård et al., 2011; Fivelstad et al., 1999, 2018; Foss et al., 2003; Hosfeld et al., 2008), use of calcium carbonate to adjust alkalinity and pH (Chen et al., 2001), magnesium deficiency (Knox et al., 1981), selenium toxicity (Bruno, 1996; Hicks et al., 1984), high density and low water flow (Damsgård et al., 2011), high levels of phosphate (Lewisch et al., 2013), and sudden changes in water chemistry with a large supply of magnesium and calcium ions (Bjerknes et al., 1994). The limitations of most of these studies were that nephrocalcinosis was an incidental finding, and it was therefore not possible to conduct in-depth investigation of its possible triggers. We therefore conducted a large-scale survey in which we collected data on as many environmental parameters as possible, including both water chemistry and feed composition, to relate

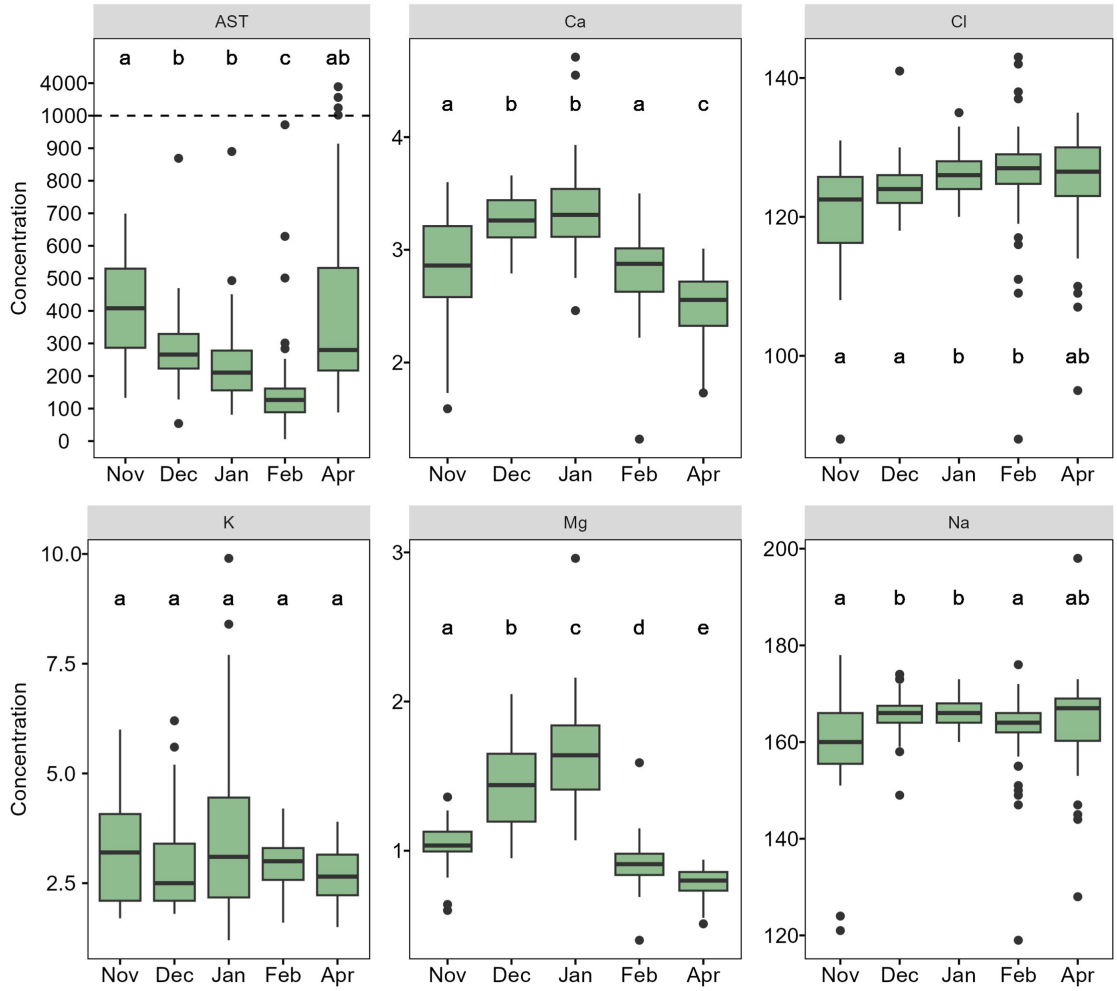

FIGURE 5 Blood chemistry parameters in Atlantic salmon at different time points during parr–smolt transformation: aspartate aminotransferase (AST), calcium (Ca), chloride (Cl), potassium (K), magnesium (Mg) and sodium (Na). Significant differences between months are indicated with different letters ($p > .05$). For AST, the y-axis is drawn together for values above 1000 (marked with the horizontal dashed line). Fish: Nov; $n = 30$, Dec; $n = 59$, Jan-Feb; $n = 60$, Apr; $n = 49$ (Tables S8–S10).

them to the prevalence and severity of nephrocalcinosis. Among those 37 parameters, only two significantly influenced the prevalence of nephrocalcinosis. These two factors were both linked to the supplementation of sea water to the production water, strongly indicating that brackish water concentrations (0.5–15 ppt) in smolt production increase the risk for developing nephrocalcinosis. We also observed that the production water in hatcheries with more than 25% prevalence of nephrocalcinosis had salinities ranging from 0.5 to 15 ppt in all but one hatchery further supporting the findings in the PLS regression model. The results obtained from the long-term survey also suggested that the prevalence and severity of nephrocalcinosis increased as response to the gradually increasing salinity of the production water.

It is a common practice in commercial production of salmon smolt to expose the fish to brackish water for several weeks prior to transfer to full-strength sea water (Lysfjord et al., 2004), but the research findings on the effects and potential benefits of this practice are equivocal (Duston, 1994; Handeland & Stefansson, 2002; McCormick et al., 1989; Saunders & Henderson, 1969) and it is clear that more research is needed on the possible negative effects of introducing brackish water (<15 ppt) during smolt production. Several studies have demonstrated that the salinity of the aquatic environment influences blood biochemical parameters of aquatic species (Jarvis & Ballantyne, 2003; Tian et al., 2020; Zarejabad et al., 2010) and that salinity fluctuations may result in osmotic stress (Goodfellow et al., 2000; Mount et al., 1997; Schwarz & Allen, 2014).

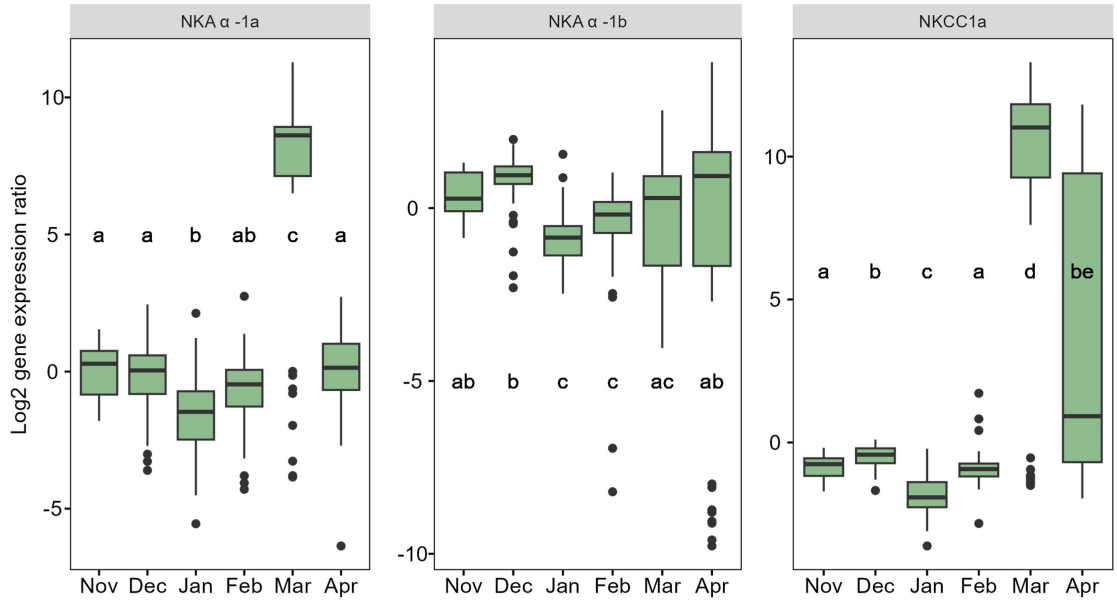

FIGURE 6 Box-and-whisker plots showing log2 qPCR expression ratios of genes related to fluid and electrolyte homeostasis in kidney tissue over time. qPCR expression ratios are normalized to the geometric mean of expression ratios of elongation factor ef1α. Na+, K+-ATPase α-1a (α-1a), α-1b (α-1b) isoform and Na+, K, 2Cl− (NKCC1a) cotransporter. Significant differences between months are indicated with different letters ($p > .05$). Fish: Nov; $n = 22$, Dec; $n = 58$, Jan; $n = 54$, Feb; $n = 44$, Mar; $n = 42$ Apr; $n = 40$ (Tables S11–S13).

In our long-term survey, the plasma concentrations of calcium and magnesium were significantly elevated in December and January in both groups compared with the other sampling points, in parallel to the observed salinity increase. This observed increase in divalent ions was comparable to what is seen after a 24-h seawater challenge test (Nieves-Puigdoller et al., 2007) and after transfer to sea. According to Urke et al. (2014), homeostasis is usually achieved within 2 weeks after exposure to sea water, but in our study the fish experienced long-term (6–8 weeks) hypercalcaemia and hypermagnesaemia causing chronic hyperosmotic stress. This could be a result of the gradually increasing salinity (Divino et al., 2016), as we observed that the calcium and magnesium concentration in plasma was reduced to homeostasis when the salinity of the production water was kept stable. An alternative explanation could be that this was a response to introducing brackish water before the fish had obtained sufficient hypo-osmoregulatory capacity. The changes in gill NKA α-1a isoform indicated that the salinity was introduced to the fish when they had not obtained sufficient hypo-osmoregulatory capacity. We also observed an increase in NKA α1*b* isoform in January, prior to the 24:0 light period, similar to that observed by van Rijn et al. (2020) in Atlantic salmon transferred to sea water. This may indicate that the salinity increase in itself promoted the changes needed for obtaining salinity tolerance in the gills, before the photoperiod cue, resulting in a mismatch in smoltification signals. In the kidney, the Na^+-K^+-$2Cl^-$ cotransporter (NKCC1a) gene expression increased in March and April compared with the other sampling points. NKCC1 has a secretory role, and the expression is higher in SW than in FW (Takvam et al., 2021). The NKA α-1a isoform was elevated in March and has previously been demonstrated to increase in SW-adapted fish compared with FW-adapted fish (Zhu et al., 2018). These changes in gene expression occurred after the addition of SW and 6–7 weeks after the start of 24:0 light period, also indicating that the salinity increase was introduced while the kidneys were still adapted to the FW environment. It is not unlikely that the prolonged duration (6–8 weeks) of osmoregulatory stress resulted in an oversaturation of the urine when the fish finally began to osmoregulate properly, which again facilitated the precipitation of calcium phosphate in the kidney.

Bakke et al. (1991) found that salmon post-smolt struggle to maintain osmoregulation in fluctuating salinities and that this becomes worse when the temperatures are excessively low or high. In our long-term survey, the salinity fluctuations occurred at low temperatures. Low temperatures could therefore have been an additional stress or for the development of nephrocalcinosis in our study.

The plasma calcium levels in our long-term survey were within normal range previously reported by Klykken et al. (2022) at all sampling points, whereas the plasma magnesium levels were outside the normal ranges in January. In Klykken et al. (2022), it was not possible to conclude whether the significantly elevated plasma calcium levels in fish with nephrocalcinosis were a cause for or a consequence of nephrocalcinosis. Based on the results of the present study, it may be both, depending on the severity of nephrocalcinosis. The fish with nephrocalcinosis in our long-term survey mainly displayed minor changes in the kidney (mild nephrocalcinosis), with little or no damages to the

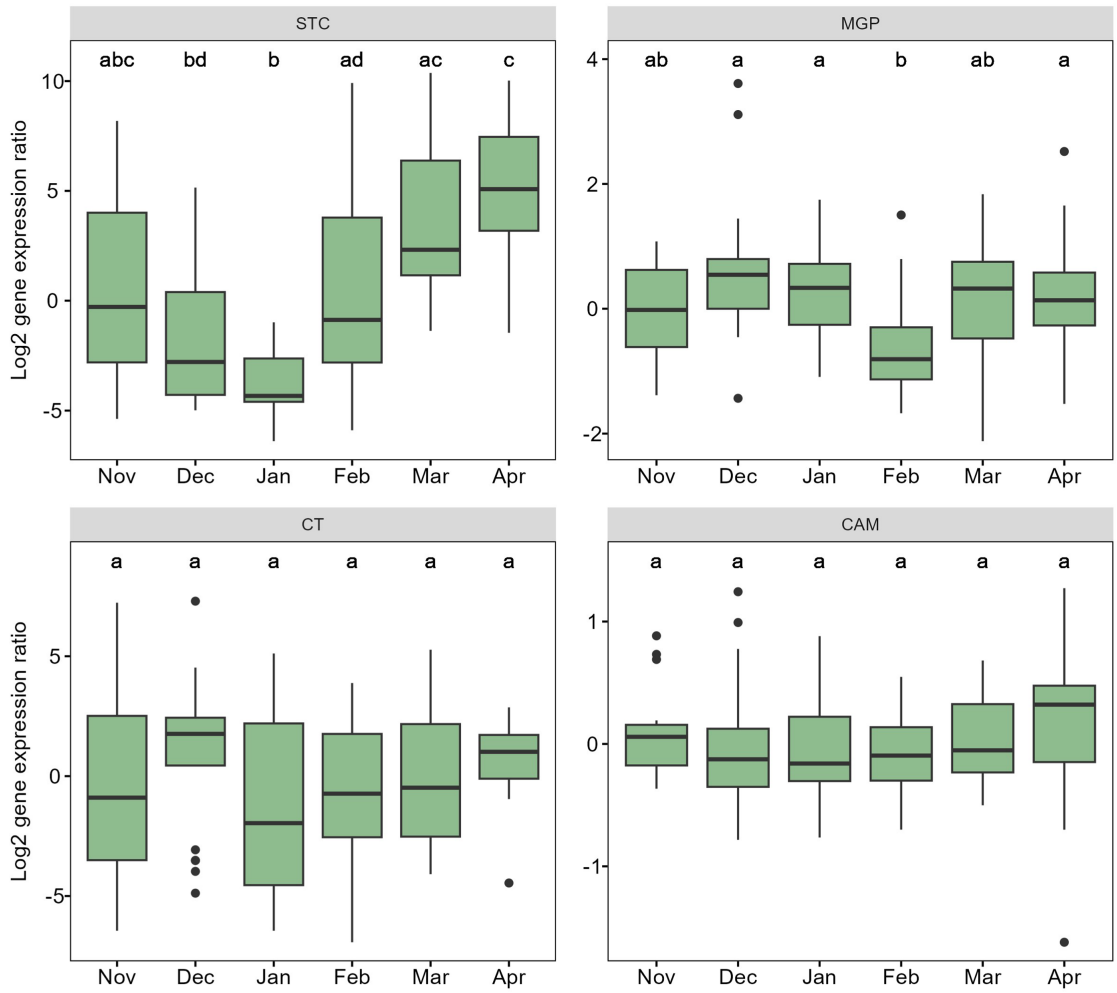

FIGURE 7 Box-and-whisker plots showing log2 qPCR expression ratios of calcium signalling-related genes in kidney tissue over time. qPCR expression ratios are normalized to the geometric mean of expression ratios of elongation factor ef1α. Matrix GLA protein (MGP), calmodulin (CAM), calcitonin (CT) and stanniocalcin (STC). Significant differences between months are indicated with different letters ($p > .05$). Fish: Nov; $n = 18$, Dec; $n = 21$, Jan; $n = 23$, Feb; $n = 27$, Mar; $n = 20$ Apr; $n = 17$ (Tables S14–S16).

kidney tissue. The plasma calcium increased prior to the increase in nephrocalcinosis prevalence and was reduced to homeostasis. If the fish had not been transferred to sea, the disease progression might have resulted in kidney damage and in turn increased plasma calcium due to failure to excrete divalent ions. The same might be true for plasma magnesium since hypermagnesaemia is linked to kidney damage (Nieves-Puigdoller et al., 2007; Singh et al., 2002). In a previous study, fish with severe nephrocalcinosis displayed hypermagnesaemia to a greater extent than fish with less severe changes in the kidney tissue (Klykken et al., 2022), supporting the view that hypermagnesaemia may be a sign of reduced kidney function. Increased levels of magnesium in our long-term survey were likely caused by the lack of sufficient hypo-osmoregulatory capacity. Both magnesium and calcium are excreted over the kidneys in teleosts (Hickman & Cleveland, 1968; Oikari & Rankin, 1985; Takvam et al., 2021), and it is possible that their elevated levels in plasma resulted in an iron overload in the kidneys. This could, in turn, have caused the aggregation of deposits which mainly consist of calcium as determined in Klykken et al. (2022). As previously pointed out, it seems that urine pH in Atlantic salmon facilitates precipitation of calcium phosphate deposits (Klykken et al., 2022). Renal mineral deposits in Atlantic salmon rarely consist of magnesium, and it might therefore be that other conditions instead of or in addition to oversaturation must be present to generate deposits with magnesium. This should be further investigated.

We did not observe differences in gene expression between fish with and without nephrocalcinosis for calcium signalling-related

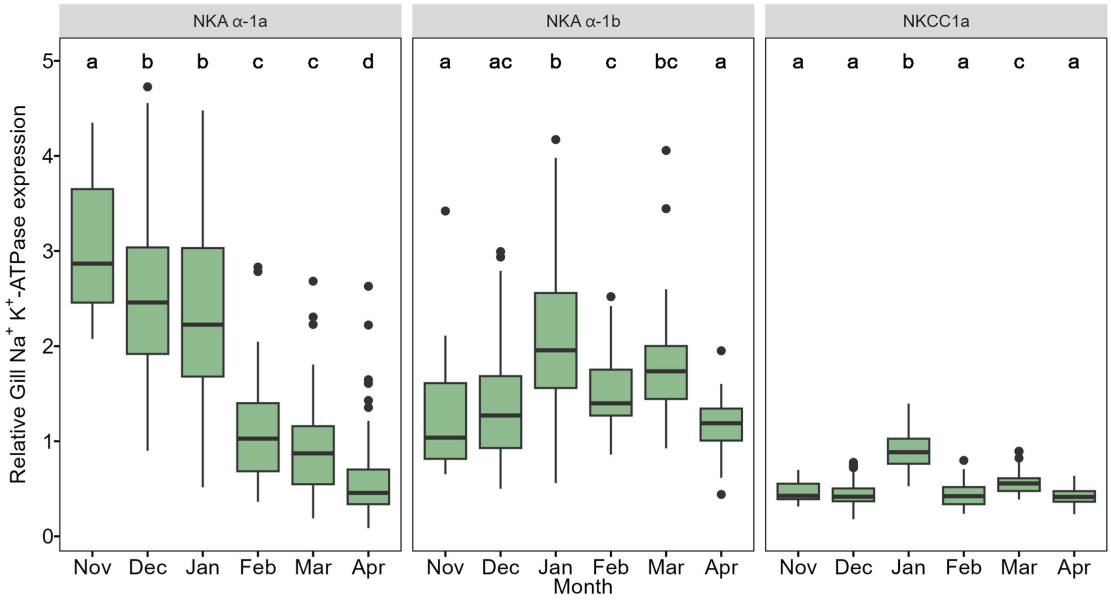

FIGURE 8 Relative gene expression of Na^+, K^+-ATPase α-1a, α-1b isoform and Na^+, K^+2Cl^- (NKCC1a) cotransporter in the gills. Significant differences between months are indicated with different letters ($p > .05$). Fish: Nov; $n = 30$, Dec-Feb; $n = 60$, Mar; $n = 52$ Apr; $n = 51$ (Tables S17–S19).

genes in the kidney. It is therefore unlikely that nephrocalcinosis in Atlantic salmon is caused by a local dysregulation of calcium homeostasis as suggested by Shavit et al. (2015) for humans. We did however find an increase in STC expression in February, March and April compared with the three previous months for both fish with and without nephrocalcinosis. STC is one of the primary hypocalcaemic hormones in fish (Amemiya et al., 2002; McCudden et al., 2001), and an increase in plasma calcium will stimulate the secretion of STC which exerts its effect on target cells in the gills, intestine and kidney (Greenwood et al., 2009). It is interesting to note that the STC gene expression increase was delayed compared with the plasma calcium increase and that the STC gene expression continued to increase after the plasma calcium levels had returned to homeostasis. It is possible that this continued 'overexpression' of STC was due to the increase in salinity since STC-producing cells have higher metabolic activity in SW than in FW (Greenwood et al., 2009; Lu et al., 1994; Wagner et al., 1998) or that it was linked to parr–smolt transformation since Domínguez et al. (2019) found differences in gene expression of STC in liver and muscle, between parr and smolts. The fact that we did not find differences in plasma chemistry and gene expression between fish with and without nephrocalcinosis may indicate that even though all fish experience osmoregulatory stress, some are able to tackle it better than others. Those who do not will develop nephrocalcinosis. Eventually, the evolution towards severe forms will cause even larger physiological changes for those individuals due to impaired kidney function. As shown in Klykken et al. (2022), the physiology of fish with severe nephrocalcinosis largely differs from that of fish with milder grades.

Taken together, the results of this study point to a causal relationship between osmotic stress induced by salinity changes and/or a challenging level of salinity combined with the fish developmental stage and previous history, and the development of nephrocalcinosis. But fluctuating salinity is not the only factor that can cause osmotic stress in salmon. In our survey, we did observe one hatchery with nephrocalcinosis prevalence above 25% which do not add sea water to their operational water. This hatchery was the only one with a production protocol where the fish were start fed in RAS and then transferred to on-growing in FT. It is possible that the high prevalence of nephrocalcinosis in this facility may be connected to transferring fish from RAS to FT, which may also result in osmoregulatory stress due to a massive change in water quality. Another example of a factor that may cause osmoregulatory stress can be high levels of CO_2 which can induce gill lesions (Fivelstad & Binde, 1994) and therefore affect the osmoregulatory capacity of individuals. Accordingly, several studies conducted on farmed Atlantic salmon have indicated that high levels of CO_2 in the water can cause nephrocalcinosis (Fivelstad et al., 1999, 2003, 2018; Khan et al., 2018). Hence, it is possible that the different parameters indicated by previous studies and observations as causative factors for nephrocalcinosis are not as contradictory as they appear, as they may all have induced osmotic stress that in turn triggered mineralization in the kidneys. In general, designing and conducting field studies in commercial facilities can be challenging as production requirements are prioritized over research considerations. Future studies on the aetiology of nephrocalcinosis should therefore include experimental induction of osmoregulatory stress by manipulation of water quality in juvenile Atlantic salmon.

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CONFLICT OF INTEREST STATEMENT

The authors declares that they have no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

All data are stored at Aqua Kompetanse AS according to internal guidelines and are available on request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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