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RESEARCH

Evaluation of novel PCR-based method to assess gill injuries in fish caused by the cnidarian *Ectopleura larynx*

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Abstract

Gill disease is a major threat to aquaculture of Atlantic salmon, with an unknown and likely underestimated contribution from cnidarians such as jellyfish and biofouling hydroids. To better understand the risk and thus enable mitigation, technology for the certain identification of cnidarian-related gill damage is needed. We used the hydroid *Ectopleura larynx* in a case study to determine whether the exposure of salmon to nematocyst-bearing hydrozoans can be deducted via non-destructive PCR-based methods. In a field experiment, we evaluated (i) whether swabbing the inside of the gill operculum in farmed *Salmo salar* and subsequent PCR analysis can provide quantifiable information about the presence of *E. larynx* material in the gill chamber and, if so, (ii) whether the screening results correlate with histological assessments of gill damage.

The developed PCR methods were able to detect the presence of biofouling hydroids in ambient water. However, despite exposure to suspended hydroid particle concentrations that did result in gill damage in some salmon, quantitative PCR results did not correlate with histological gill assessments. For opercular swabs to serve as a diagnostic tool for detecting biofouling-mediated gill damage in live salmon, increased specificity of genetic markers and improved sampling methods are needed.

Animal trial permit no. 24252 (granted 06.07.2020, Norwegian Food Safety Authority).

Keywords Gill disease · Atlantic salmon · *Salmo salar* · Biofouling · Net cleaning

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Introduction

Aquaculture of Atlantic salmon (*Salmo salar*) is one of Norway's key industries with a production volume of 1.5 M metric tons valued at NOK 76 B in 2021 (Norwegian Directorate of Fisheries 2022). To realise its goal of a significant increase in production over the coming years, the industry needs to overcome a multitude of challenges, including those related to fish health and welfare. In 2022, the average mortality of farmed salmon at sea was 16.1% (56.7 M individuals). Gill disease is considered to be the most important cause for reduced fish growth and welfare, and increased mortality, with a rising tendency (Sommerset et al. 2023). Gill health in salmon can be impacted by many factors such as water chemistry, husbandry practices, pathogens, harmful algal blooms, and gelatinous zooplankton (Boerlage et al. 2020).

Jellyfish, pelagic life stages of cnidarian hydrozoans and scyphozoans, can harm fish indirectly by clogging the net and impeding water flow through the pen (Clinton et al. 2021a). They also pose a direct threat to fish health through their nematocysts—stinging cells that can deliver venom and are usually used for hunting prey and defence (Cegolon et al. 2013). Exposure of salmon to nematocyst cells has repeatedly been shown to cause serious injury (Baxter et al. 2011; Marcos-López et al. 2016; Mitchell et al. 2011; Småge et al. 2017). When jellyfish enter aquaculture net pens, either intact or fragmented following passage through the mesh, fish have been reported to undergo respiratory distress, loss of appetite, lethargy, and/or increased jumping behaviour. Moreover, direct contact with fish has been shown to cause traumatic damage, impaired gill function, and initiation of secondary disease through injuries to skin, particularly, gill tissues (Marcos-López et al. 2016; Mitchell et al. 2011; Småge et al. 2017; and reviewed in Clinton et al. 2021a). Jellyfish are thought to cause at least 4% of fish deaths and injuries in Norwegian salmon farms (Sommerset et al. 2023). In addition, the nature of injuries reported for some high-mortality events within wellboats implicates cnidarians as causal agents.

Cnidarians and their impacts are not a notifiable disease and are not part of national screening programmes. There is also no readily available technology to identify whether gill damage stems from cnidarians (Sommerset et al. 2023). As a consequence, the full impact of nematocyst exposure on the Norwegian aquaculture industry has not been quantified. This is particularly important given that also husbandry practices, such as net cleaning of salmon pens, are a potential cause for gill injuries (Sommerset et al. 2022). Net cleaning is conducted regularly (up to weekly) in Norwegian salmon farms to remove biofouling organisms to secure water flow through the pen and avoid other health-related challenges (reviewed in Bannister et al. 2019; Bloecher and Floerl 2021). During net cleaning, biofouling organisms or their fragments are released into the cage environment, where farmed fish are exposed to them (Carl et al. 2011; Elsheshtawy et al. 2023). One of the most abundant biofouling taxa on Norwegian salmon farms are hydroids—nematocyst-bearing close relatives of jellyfish (Guenther et al. 2010; Schuchert 2010). As for jellyfish, farmers report reduced appetite, gill bleeding, and general signs of stress in salmon during net cleaning. Laboratory experiments have shown that particles of the ubiquitous biofouling hydroid *Ectopleura larynx* can injure salmon gills, resulting in damage that is visible for up to 7 days (Baxter et al. 2012; Bloecher et al. 2018). Recent field sampling confirmed laboratory results through observation of subacute vascular gill damage (thrombi) in salmon following net cleaning (Østevik et al. 2021).

Farming practices such as pen net cleaning, and environmental change associated with warming climates likely increase the exposure of farmed fish to cnidarian stinging cells

(Attrill et al. 2007; Clinton et al. 2021a; Holst 2012). Practical and cost-effective diagnostic tools are needed to enable detection and quantification of nematocyst-related health incidences in Norwegian and global finfish aquaculture. One option for such a tool is the use of swab samples in combination with genetic analysis such as polymerase chain reaction (PCR). This method is already widely employed to detect the presence of high-risk pathogens such as the parasitic amoeba *Paramoeba perurans*, the causative agent for amoebic gill disease (AGD) (Clinton et al. 2021b; Downes et al. 2017). Such assessments are based on non-destructive sampling of the gill, and thus provide a clear advantage over mortal sampling for histological analyses, particularly in the case of large-scale surveillance and monitoring. There are, however, some concerns that swabbing can injure the extremely sensitive gill filament tissues by causing focal loss of lamellae and sloughing of epithelial cells (Mitchell et al. 2023). To avoid inadvertent health and welfare risks, alternative swab locations may be a potential solution. When hydroid or jellyfish fragments enter the gill cavity of farmed salmon, nematocysts may be activated and/or traces of cnidarian tissue become entrained in the mucus layer coating the walls (Baxter et al. 2012). Non-destructive sampling techniques targeting gill cavity mucus may be a method for determining whether a fish's gills have been exposed to harmful cnidarians, and act as a 'warning system' for gill damage resulting from such exposure. While exposure to planktonic jellyfish is hard to predict, contact with biofouling hydroids is highly predictable and a regular occurrence for many Norwegian salmon farms (Bloecher and Floerl 2021; Guenther et al. 2010). This makes it a suitable model system for evaluating the efficacy of alternative detection tools.

This study used the hydroid *E. larynx* as a case study to determine whether the exposure of salmon to nematocyst-bearing hydrozoans can be deducted via non-destructive gill swabs. A field experiment was conducted to evaluate: (i) whether swabbing the inside of the gill operculum in farmed *S. salar* can provide quantifiable information about the presence of *E. larynx* material in the gill chamber and, if so, (ii) whether the screening results correlate with histological assessments of gill damage. The study was carried out at a commercial salmon farm located in Mid Norway. It included quantitative assessment of biofouling assemblages on pen nets and particle loads within the pen environment caused by net cleaning.

Materials and methods

Ethics statement

The study was carried out in accordance with the EU Animal Welfare Act and the Norwegian Regulations on the use of animals in research. The experimental protocol was approved by the Norwegian Food and Safety Authority (Permit. No. 24252).

Experimental site and fish

The experiment was conducted at Tristeinen, Mid-Norway (63.87° N, 9.62° E), a dedicated full-scale research farm site (SINTEF ACE) operated commercially by SalMar AS. The site consisted of seven net pens carrying nylon nets treated with a green, non-biocidal coating to ease cleaning. Pens had a circumference of 157 m, tapering to 25 m in depth after 12 m of straight net wall, and were arrayed in two parallel rows. All pens were equipped with protective lice skirts (Jónsdóttir et al. 2023) in the upper 7 m.

Net cleaning was conducted by a service provider and as part of a routine 14-day schedule. The last cleaning event had taken place 13 days prior to the experiment, on 17.9.2020. Sampling was conducted in association with two consecutive cleaning events on 30.09.2020 and 15.10.2020 in two net pens per event (pens A and B, and pens C and D, respectively). All fish in these four pens were of the same genetic strain and origin. They had been transferred to sea in July 2020 and by the time of sampling had an average weight and length of 603 g and 35 cm, respectively.

Assessment of biofouling on nets and particles in water column

Before net cleaning took place, biofouling was quantified in the following ways:

1. Visual census of biofouling communities on the net walls: Two vertical transects were filmed along the net approx. at the north and south sides of the pen. The sampling rig consisted of a metal frame that could be manoeuvred up and down the pen nets via a tether. The frame had a GoPro Hero 5 camera and a 300 lm LED lamp mounted to it and provided a constant focal distance of approx. 40 cm to the net wall. For each transect, three replicate images showing approx. 30×16 mesh openings were captured at both 2 m and 10 m depths ($N = 12$/pen).
2. Suspended particle concentration measurements (pens A and B only): To assess the particle load in the pens, images were taken using a particle imaging system ('SilCam') as described by Davies et al. (2017). SilCam images were recorded for approximately 15 min at depths of 3 m and 10 m (i.e. within and below the lice skirt-protected net volume), at a rate of 15 frames per second and using a ×0.125 telecentric lens. This equated to sampling a total volume of 0.2 L per image (3 L per second) and the ability to resolve particle sizes from 0.1 to 50 mm.
3. Plankton samples: To assess the particle load and composition, three replicate samples were taken with a plankton net (100 µm, 30 cm diameter) from 10 m depth to the surface (sampling volume ~ 0.7 m^3) at the down-stream side at the inside of each pen, at approx. 1.5 m distance to the net wall. Samples were preserved with 4% formalin ($N = 12$).

Net cleaning

Cleaning was conducted by a service provider using a FNC 2.0 net cleaning rig (Akva Group, Norway). The cleaner operated at 95 to 150 bar of water-jet pressure and took approx. 2 h to clean each pen net. Plankton sampling and SilCam imaging were repeated during net cleaning, starting 30 min into the operation, using the methodology described above.

Sampling of fish gill and gill cavity tissues

In each pen, 15 fish were sampled with opercular swabs before, during (approx. 1 h into the operation), and 1 day after the net cleaning operation (total $N = 180$). Fish examined before and after cleaning were also sampled for histological analysis (total $N = 120$). Fish were attracted to the surface using feed, lightly crowded in a net (max. crowding stage 2 according to Noble et al. (2018)) and transferred into a tank with seawater and anaesthetic using a hand-held knotless dip net.

Fish sampled before cleaning on 30.09.2020 and a day after were directly euthanised using an overdose of anaesthetic (Benzorion Vet, Orion Pharma Animal Health, 0.14 mg/L) before samples were taken. On 15.10.2020, sampling was conducted in connection to standard husbandry practices on site where several fish were sampled and returned to the pen. Therefore, fish were anaesthetised (Benzorion Vet, 0.08 mg/L) before the subsample group for the experiment was euthanised in a bath with an overdose of anaesthesia (Benzorion Vet, 2.5 mg/L). During both sampling events, weight and length of fish were recorded before the following samples were taken consecutively from each sampled fish:

1. Opercular swab: Taken from the inside of the left gill operculum by swiping the two 'sides' of a sterile SafeCollect swab (Zymo Research) each three times back and forth (covering an area of approx. 2 cm^2). The swab tip was transferred into a DNA/RNA Shield Lysis & Collection Tube (Zymo Research) and stored at 4 °C until further processing.
2. Histology sample: The second gill arch on the left side was excised for histological analysis (within 5 min of euthanasia). The samples were preserved in 10% buffered formalin.

Fish sampled during net cleaning were only sampled with opercular swabs as histopathological changes were expected to take longer time to develop (Bloecher et al. 2018). For this non-invasive procedure, fish were anaesthetised (Benzorion Vet, 0.08 mg/L), weight and length measured, swab sampled, and released back into the pen after a supervised recovery period.

Sample analysis

Pen net imagery

Biofouling on net wall images was analysed according to the guidelines described in Bloecher and Floerl (2018). In short, the image was overlaid with a line grid that intersected with the net in 59 or more instances (average 70 intersections). For each point, the presence/absence of fouling, identified to key taxonomic groups, was determined.

SilCam images

An image analysis script was developed using Python (v3.6) and OpenCV (v3.4.2) and iterated through the SilCam images in the same order as they were acquired. For each image, an average of the ten previous images was calculated in order to generate an estimated image of the background; this image was then subtracted from the first image in order to isolate all the particles from the background. The particles were then segmented out by thresholding the image on pixel intensity, where pixel values of more than or equal to 12 were defined as a part of a particle. Each individual particle was then extracted from the segmented image using OpenCV function 'findContours', where an ellipse fitting function could be implemented to retrieve the major axis for each individual particle. Data for each individual detected particle along with the timestamp were stored in separate files for further analysis.

Plankton sample

Samples were gently rinsed in freshwater to remove formalin and decanted through a 100-µm sieve. The content was resuspended in a Bogorov chamber to be analysed under a dissecting microscope. All hydroid particles in the sample were examined and allocated into the following categories: (1) individual hydroid; (2) hydroid colony (2–10 individuals); (3) other hydroid particles such as hydranths, gonophores, tentacles, and hydrocauli. In addition, the presence of other major taxonomic groups as well as coating particles was noted.

Swab samples

Genetic marker (swab) analysis: Total DNA was extracted from operculum swab samples using ZymoBIOMICS DNA Miniprep Kit according to manufacturer's protocol. Total DNA was eluated in 50 µL PCR-grade H_2O and subjected to quality control using nanodrop1000 (Thermo Fisher Scientific), and quantification using Qubit 3.0 (Thermo Fischer Scientific) and Qubit dsDNA HS assay kit (Invitrogen).

To analyse abundance of *E. larynx* in operculum mucus samples, copy numbers of the *E. larynx* cytochrome oxidase subunit 1 (COI) gene (GenBank accession nr.: MG935196.1) were quantified using digital PCR. Corresponding forward primer (5′-CAGATATGGCGT TTCCCCGA-3′), reverse primer (5′-GAACCACCGGAATGGGTGAT-3′), and Taqman probe (5′-HEX-AGAAGGAGCTGGAACCGGTTGAACA-3′) targeting a 157-bp internal fragment of the COI1 gene were designed using Primer3 software tool (NCBI). The detailed dPCR workflow was performed as described elsewhere (Netzer et al. 2021). For each sample, a 25-µL reaction mixture was prepared (1× concentrated PerFecTa Multiplex qPCR ToughMix (Quanta Biosciences), 1 µM fluorescein, 1 µM of primers, 250 nM of corresponding TaqMan probe, and an appropriate amount of total DNA as template) and loaded on a Sapphire chip. Sample partitioning and PCR were performed in Sapphire chips in the Naica Geode using the following program: (1) 50 °C for 2 min, (2) 95 °C for 5 min, (3) 95 °C for 15 s and 57 °C for 30 s, 50 cycles. Data analysis was performed using Crystal Miner software V2.3.5 (Stilla Technologies). Reactions with no template (NTC) were performed to control for DNA contaminations. In addition, total DNA extracted from tank water from a commercial freshwater RAS facility was used as a negative control for the presence of *E. larynx*.

Gill histology

Gills were fixed in 10% buffered formalin (4% formaldehyde, 0.08 M sodium phosphate, pH 7.0), processed routinely, and sections were stained with haematoxylin-eosin (HE) and scanned for histopathologic examination as previously described in Østevik et al. (2021). Slides were randomized using computer-generated random numbers, and the pathologist was 'blinded' regarding information on pen, time point, and results of other analyses. A two-step assessment system was used to evaluate the extent and types of gill pathology present (for details, see Supplementary information and Østevik et al. (2021)). In short, first the number of lamella available for evaluation in each sample was estimated by counting the number of filaments and classification of their plane of section as good, medium, or poor. Then, all lamellae with hyperplasia, necrosis, thrombi (subacute vascular lesions), acute aneurysms, haemorrhages, and/or reactive and reparative (chronic) vascular lesions

were counted. These counts were used to calculate the estimated percent of gill tissue affected for each type of lesion. In addition, the presence or absence of the following lesions and any pathogens and possible pathogen-associated lesions present were recorded as 0 or 1 (dichotomous variables): epithelial cell necrosis; adhesion of lamella; hypertrophy/swelling of epithelial cells; lamellar oedema/'lifting'; inflammation of the filaments. One sample taken from Pen B after net cleaning was excluded from further analysis due to the presence of bacteria and necrotic debris in the sample.

Statistical analysis

Genetic markers Fisher's exact test was used to compare the prevalence of positive hydroid swab samples before net cleaning to the prevalence observed during net cleaning (before vs. during) or the day after net cleaning (before vs. after). Comparisons were carried out individually for each pen.

Histology Permutational analysis of variance (PERMANOVA, Primer v.7) was used to compare the total abundance of vascular lesions per lamella, as well as the abundance of thrombi, acute lesions, and haemorrhages between samples taken before and after net cleaning (factor 'Cleaning': Before cleaning' and 'After cleaning', fixed) sampled from the four pens ('Pen': pens A–D, fixed). Comparisons were based on Euclidean distance and 9999 unrestricted permutations of residuals under a reduced model with a significance level of 5%. Where the number of unique permutations was < 100, the Monte Carlo asymptotic pMC-value was consulted. Where PERMANOVA indicated no significant differences between factors (significance level $\geq 25\%$), the term was pooled to increase power (Anderson, 2017; Anderson et al. 2008).

Results

Biofouling cover

Biofouling cover varied between the four pens as well as between the two depths and orientations. Overall average biofouling cover was 64%, with the highest abundance in pen B (78%), followed by pens C (69%), D (67%), and A (43%) (Fig. 1). All pens showed a tendency for higher biomass at 10 m depth compared to 2 m depth. Species composition differed between depths, at 2 m depth consisting predominantly of algae, whereas samples collected at 10 m depth consisted predominantly of the hydroid *Ectopleura larynx* and caprellid crustaceans.

Particle concentrations

Net cleaning led to a consistent increase in suspended particles of most size classes examined via SilCam imagery (Fig. 2), the only exception being particles < 2 mm in pen B. While differences between particle concentrations in the 3 m and 10 m depths were minimal before cleaning, cleaning led to markedly higher concentrations (up to fourfold) at 3 m depth within the lice skirt volume than below at the 10 m depth. In general, average

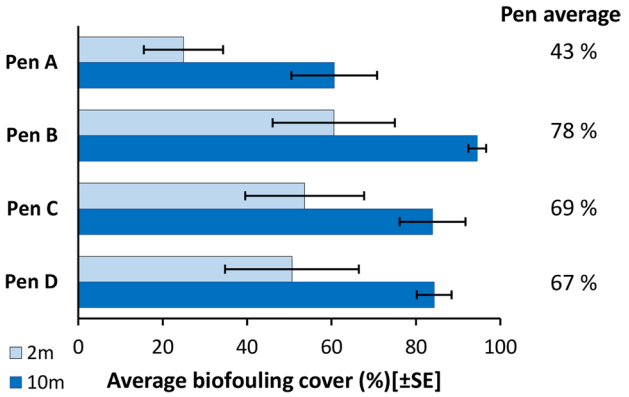

Fig. 1 Biofouling cover. Average biofouling cover (% ±SE) measured at two depths (2 m vs. 10 m, $N=6$), and per pen

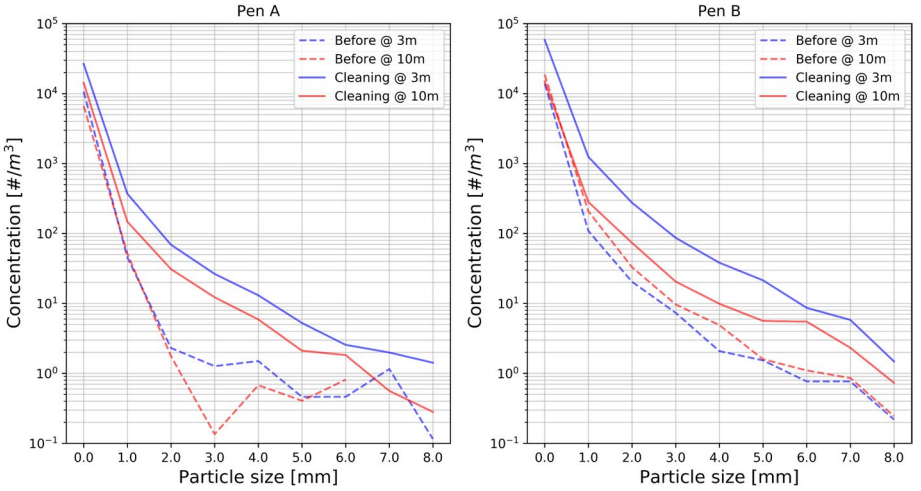

Fig. 2 Total particle concentration. Results from SilCam analysis: Average particle concentration (log scale) per size in pens A and B comparing two depths (3 m: within lice skirt volume (solid line) vs. 10 m: below lice skirt (broken line)) before and during net cleaning for a 15 min measurement interval

particle concentration decreased with particle size, and particle abundance was higher in pen B than in pen A.

Similar trends were measured in the plankton samples. While absolute concentrations of hydroid particles differed between the four pens, they all increased profoundly—approximately 31-fold—during net cleaning (Fig. 3), from an average of 6.7 ± 2.9 particles m^{-3} before cleaning to 177.8 ± 40.8 particles m^{-3} during cleaning. Before cleaning, suspended biofouling particles mostly consisted of hydroid fragments, while during cleaning entire hydroid polyps were found in the samples.

In addition, every plankton sample taken during net cleaning contained green, non-biological particles consistent with the coating present on the net. These were absent in the samples taken before cleaning.

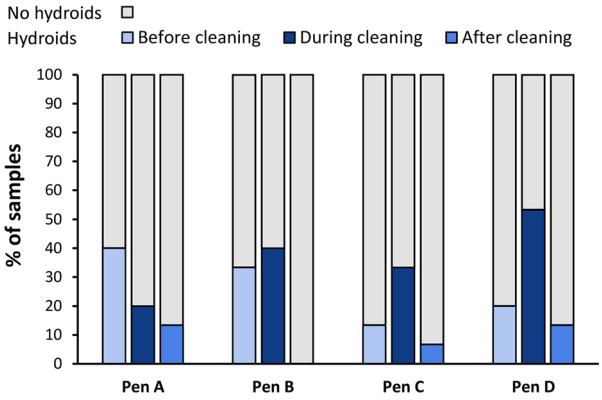

Fig. 3 Hydroid particle concentration. Results from plankton sampling: Bars denote average number of hydroid particles per m^{-3} in four pens ($N=3$), sampled before and during net cleaning, with individual measurements depicted as points

Histology results

Overall gill health of the 119 fish included in the assessment was good both before and after net cleaning. The average number of lamellae with any vascular lesions (acute, subacute, or chronic, including haemorrhages) ranged from 0.15 to 3.57% with an average of 0.63% and was not affected by net cleaning, yet differed between pens (Pen: $f_{3,118}=3.781$, $p<0.008$). With on average 0.84% of lamellae affected, fish sampled from pen B had significantly more lesions than fish from pens C and D (0.48% and 0.55%, respectively). There were no differences to pen A (0.65%) or among the other pens.

Among the recorded lesions, the occurrence of subacute vascular lesions (i.e. thrombi, suggestive of damage caused by cnidarians) was the only gill health variable significantly affected by net cleaning (Fig. S1 in Supplementary information). Here, the number of lamellae with thrombi increased after net cleaning in two out of the four pens (Cleaning ×

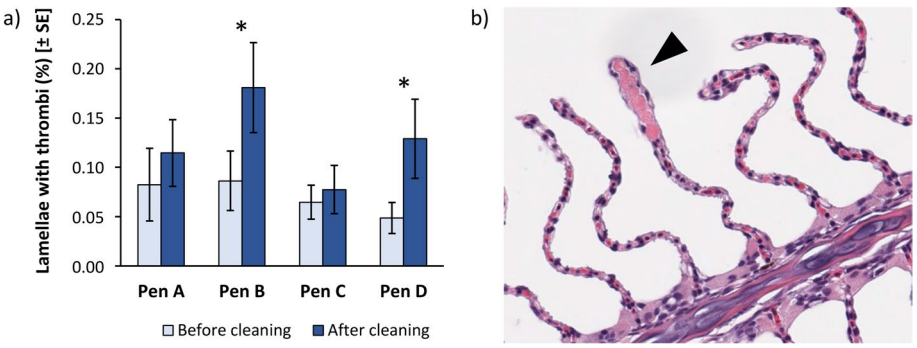

Fig. 4 Gill histology results. **a** Average number of thrombi per lamella (% ±SE) in samples taken before and 1 day after net cleaning took place ($N=15$). The asterisk indicates significant differences within pens comparing before and after net cleaning ($p<0.01$). **b** Example of a thrombus in a gill lamella

Pen, $f_{3,118}$=3.422, $p<0.020$), doubling in pen B from 0.09 to 0.18% and tripling in pen D from 0.05 to 0.15% (Fig. 4).

Throughout the four pens, signs of amoebic gill disease (AGD) were found in 16 fish and intracellular bacteria (epitheliocysts) and multicellular parasites consistent with metacercaria were present in gills of four and 20 fish, respectively.

PCR-based gill mucus analysis

A TaqMan® digital PCR assay was developed and verified using total DNA extracted from *E. larynx* samples as positive control. The assay revealed high linear dynamic range over 4 log10 dilutions with a regression coefficient of $R^2=0.9999$. A total DNA concentration of 1.66 pg/µL 47 COI gene copies were detected, demonstrating high sensitivity of the assay. In total, DNA from 180 opercular swab samples was extracted and subjected to quantification of the COI gene copy numbers employing dPCR. In 43 samples, low concentrations of the COI marker gene were found with abundances varying from 0.14 to 9.47 copies/µL DNA extract, while 137 samples tested negative.

The prevalence of a positive swab sample obtained from mucus associated with salmon gill opercula did not correlate with the increasing number of hydroid particles in the water, or the evidence of gill damage following exposure to hydroids. Neither during net cleaning nor on the day after was the prevalence of hydroid-positive swab sample significantly increased compared to before cleaning in any of the four sampled pens (Fisher's exact test, $p>0.05$; Fig. 5). In contrast, the only significant difference observed was found for pen B, where significantly more positive samples were detected before cleaning than on the day after (5 vs. 0 fish, respectively; $p=0.042$).

Discussion

Our study again demonstrates the tremendous release of biofouling particles during salmon pen net cleaning, and the potential for gill injury in salmon exposed to these particles. We also show that PCR methods are able to detect the presence of biofouling hydroids in ambient water using low-impact swab samples of gill mucus. However, despite exposure to high suspended hydroid

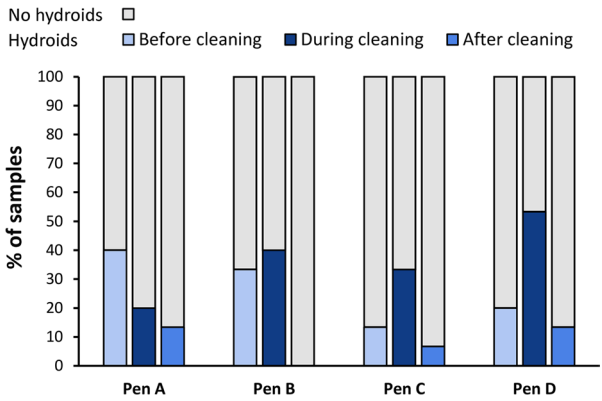

Fig. 5 Swab sample results. Prevalence of gill operculum swab samples with positive hydroid identification ($N=15$ per sampling time and pen)

particle concentrations that did result in gill damage in some salmon, quantitative PCR results did not correlate with histological gill assessments. At this stage, opercular swabs are unable to serve as a diagnostic tool for detecting biofouling-mediated gill damage in live salmon.

Biofouling and gill damage

The hydroid *Ectopleura larynx* dominated the net pen walls below the lice skirt in all four pens. While hydroids release particles such as hydranths as part of their life cycle also without being agitated through a net cleaner (Pyefinch and Downing 1949), net cleaning caused a 31-fold increase in abundance of hydroid particles, thus increasing exposure risks for the fish. Gill injuries in the form of subacute vascular damage (thrombi) increased after net cleaning in fish from two of the four pens. The presence of thrombi is suggestive of gill injuries after contact with *E. larynx* and has been observed in Atlantic salmon during both laboratory and field experiments, persisting for up to 7 days post-exposure (Bloecher et al. 2018; Østevik et al. 2021). The formation of thrombi is likely the result of toxins released from cnidarian nematocysts (Helmholz et al. 2010; Lecaudey et al. 2024), and the level of exposure determines the magnitude of associated gill injuries.

Effects on gill health were restricted to pens that had high initial biofouling cover and high particle release, a similar pattern to that described in Østevik et al. (2021). In fish with gill damage, only ~ 1% of gill lamellae were affected, which is unlikely to result in clinical disease such as respiratory failure. However, repeated exposure to cnidarian nematocysts may result in an accumulation of lesions if recovery periods between cleaning events are shorter than repair mechanisms require. While assessment 8 days post exposure did not reveal damage in field sampling (Østevik et al. 2021), earlier laboratory experiments showed that thrombi may persist for up to 7 days following exposure (Bloecher et al. 2018). With cleaning scheduled as frequent as every 5 days on some sites during the main biofouling season, and further repeat exposure during cleaning of neighbouring pens, the cumulative risk of gill damage may be higher than what is apparent from our data. This cumulative risk may also include the likelihood of secondary infections (Elsheshtawy et al. 2023), similar to what was thought to have contributed to a tenacibaculosis outbreak in a Norwegian salmon farm following exposure to the jellyfish *Dipleurosoma typicum* (Småge et al. 2017).

A lack of effect in pens A and C may also have been caused by sampling bias. We used feed to attract fish to be caught for sampling—and sampling hence relied on fish appetite (Thorvaldsen et al. 2019). However, fish farmers regularly report lack of appetite and feeding in salmon during and after net cleaning, often for up to 8 h (SINTEF, unpublished data). Lack of appetite has also been observed in salmon that incurred gill injuries following exposure to the jellyfish *Pelagia noctiluca* (Marcos-López et al. 2016). As such, our feed-based sampling may have selectively targeted fish that had not been exposed to harmful biofouling particles, had no gill damage, and, as a result, had retained their appetite.

The lack of significant effects on the gills other than those suggestive of cnidarian insults may indicate that the other organisms released into the pen volume during cleaning (mainly various algae, bryozoan particles, and coating particles) did not affect gill health, or were not present at sufficient densities for causing harm. If the former, then there may be a seasonality to the risk for gill injury in connection to net cleaning, based on the growth season of hydroids in Norway. While there exists survey data on the cause for fish mortality and the occurrence of specific diseases (Sommerset et al. 2023), the complex aetiology of gill disease (Boerlage et al. 2020; Marcos-López et al. 2016) as well as the lack of details in the reporting makes

it impossible to attribute these observations to cnidarian-related damage and thus search for temporal or spatial trends that could be correlated to biofouling growth patterns. There is, however, a general trend in Norway with fish mortality being higher in the southern farming regions than the northern ones (Sommerset et al. 2023), potentially reflecting a contribution of a longer and more abundant occurrence of biofouling in the warmer southern waters.

The presence of coating particles in every single sample taken during cleaning underscores that current net cleaning practices are not sustainable for coated nets as they likely result in contamination (Bloecher et al. 2019; Floerl et al. 2016). While the coating used in this experiment did not contain biocides, damaging it diminishes its functionality, potentially requiring increased cleaning efforts—and thereby increasing greenhouse gas emissions (Nistad et al. 2021) and the release of microplastics (Krüger et al. 2020).

Effects of the lice skirt on particles in water

Concentrations of cleaning waste particles in water were up to four times higher within the lice skirt volume at the 3 m depth than below the skirt at the 10 m depth. This can be explained by the shielding effect of the lice skirt (Jónsdóttir et al. 2023) which can cause a reduction in water flow through the pen by as much as 91% in low-current environments (Frank et al. 2015). While information on effects of such extended retention times of particles in the shielded volume on fish health is currently lacking (reviewed in Jónsdóttir et al. 2023), some farmers do report increased difficulties with gill health in pens with skirts (Misund et al. 2020). Particle concentrations in this study were higher within the skirt volume, and are expected to last longer due to the decreased water exchange (Jónsdóttir et al. 2023). The presence of lice skirts during net cleaning may thus represent an additional risk factor for gill health that should be assessed further.

Use of PCR-based mucus swabs

While our assay showed a high sensitivity to *E. larynx*, no correlation to individuals with histopathologic evidence of hydroid damage could be established. A potential explanation for this could be that the COI gene copy numbers detected were close to the detection limit or not detectable at all. However, considering that one hydroid particle may contain several COI gene copies, significantly higher COI gene copies were expected to be found in mucus from fish with gill damage symptoms after net cleaning. It is possible that mucus from the operculum is not a suitable location for retrieving hydroid particles or nematocysts. As the main passage of water during breathing is first through the gills before reaching the gill operculum, gills may filter out some of the waterborne particles, thus decreasing chances of detection in mucus of the gill operculum. Moreover, the multilayered structure of the gill lamellae may provide better retention of hydroid particles compared to the relatively smooth opercular surface, thus increasing chances for collection on a swap and facilitating detection. Future experiments that collect mucus directly from the gill tissue should be considered to further assess the suitability of gill mucus as sample material.

Our dPCR assay was developed based on the COI gene sequence (Accession nr. MG935196.1) from an *E. larynx* sample collected off the Swedish coast (Västra Götaland County). Even though it displayed high sensitivity and specificity, the COI gene sequence

should be analysed for local or regional (Norway-based) populations of *E. larynx*, and corresponding oligonucleotide sequences optimised as needed. Potential further evaluation of a PCR-based assay should also include laboratory-based exposure studies with different concentrations of hydroid particles. This would allow for more differentiated diagnosis of gill damages.

In its present format, the digital PCR assay used in our study is not suited as a screening tool for hydroid-mediated gill damage in farmed salmon. However, non-invasive swab methods remain attractive options for diagnostic tools for nematocyst damage and should be further examined. Increasing specificity of genetic markers as well as improving sampling methods will be an important step towards a functioning assay. This is particularly relevant given the anticipated increase in cnidarian proliferation, distribution, and abundance associated with climate change (Attrill et al. 2007; Clinton et al. 2021a; Holst 2012).

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Author contribution Nina Bloecher, Roman Netzer, and Oliver Floerl conceived and designed the study. Nina Bloecher and Rolf Sivertsgård conducted the field experiment. Nina Bloecher, Liv Østevik, Oliver Floerl, Rolf Sivertsgård, Marianne Aas, Bjarne Kvæstad, Deni Ribičić, and Roman Netzer were involved in sample analysis and data processing. The first draft of the manuscript was written by Nina Bloecher. All authors commented on previous versions of the manuscript and read and approved the final manuscript.

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Data availability The data that support the findings of this study are available upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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