Aquaculture 582 (2024) 740455

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Exploring the impact of thermal delousing on gill health and microbiome dynamics in farmed Atlantic Salmon

Ahmed Elsheshtawy [a,b], Benjamin Gregory James Clokie [a], Amaya Albalat [a], Are Nylund [c], Birgit Lilletveit Kvåle [c], Linda Andersen [c], Lindsey Jane Moore [c], Simon MacKenzie [a,*]

[a] Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, UK
[b] Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University, Kafr El Sheikh City 33516, Egypt
[c] Fish Disease Research Group, Department of Biological Sciences, University of Bergen, Bergen 5020, Norway

ARTICLE INFO

Keywords:
Aquaculture
Gill microbiota
Immune response
Histopathology
Thermolicer
Dysbiosis

ABSTRACT

The economic cost of salmon louse (*Lepeophtheirus salmonis* and *Caligus rogercresseyi*) infestations in the Atlantic salmon (*Salmo salar*) industry has been estimated to be around $900 million annually. This high cost has driven a concerted effort to develop, examine, understand, and implement various methods for louse control. Husbandry interventions utilising warm water exposure have been highly successful in complementing traditional chemotherapeutants, especially as the efficiency of the latter has reduced in recent years. In this study, we sought to examine the impact of thermal delousing on gill health in two commercial sites with different historical husbandry and treatment interventions prior to and post-thermal treatment. Methods to characterise gill health and the response to thermal treatment included a detailed examination of the gill for microparasites using both histology and qPCR and targeted immune gene expression analysis, most notably antigen-presenting cells (*mhc ii*), proinflammatory cytokines (*il-1β* and *tnf-α*) and inhibitory cytokines (*tgf-β* and *il-10*). Furthermore, we examined the bacterial communities present on the gill surface using 16S rRNA amplicon sequencing. Data obtained from these trials indicated a minimal impact on gill microparasite prevalence in response to the thermal treatment. The expression of immune markers exhibited a significant decrease across both sites after treatment. Intriguingly, marked differences in the gill bacterial communities in response to treatment between the two sites were clearly observed. This divergence could be attributed to the notable differences in husbandry history and health status of the fish at the two sites prior to the thermal treatment. Our data suggest that microbiome diversity is an informative indicator of fish gill health and could be used to define appropriate interventions when treating sea lice.

1. Introduction

The economic and ecological impacts of salmon louse infestation in the Atlantic salmon farming industry is a significant challenge to sustainability and to animal health and welfare. The salmon louse (mainly *Lepeophtheirus salmonis* in the northern hemisphere and *Caligus rogercresseyi* in Chile) is a marine ectoparasitic copepod, belonging to the family *Caligidae*, infesting farmed and wild Pacific and Atlantic salmonids (Berland and Margolis, 1983). *L. salmonis* undergoes a direct life cycle consisting of eight distinct stages (Hamre et al., 2013). The cycle begins with three free-living stages: two planktonic nauplii and one infective planktonic copepodid, which dedicates it's time to seeking a host. Once the copepodid locates a host, the five parasitic stages commence, with two non-motile chalimus embedded on the skin of the salmon, followed by two motile pre-adults and one adult stage. These mobile stages move freely over the salmon's skin and feed on mucus, skin, and blood using their rasping maxillae. Preferred attachment sites for lice are mainly the fins and skin around the head, nonetheless, attachment to the gills and premature feeding on blood has been recently reported (Heggland et al., 2020). The primary pathology arises from the mechanical damage and feeding behaviour of the parasitic stages. Lice infestation can be moderate or high, resulting in skin erosions, body fluid and blood loss, reduced growth, and primary stress responses (Pike and Wadsworth, 1999). Moreover, lice secrete cathepsin L, trypsin, and prostaglandin E2, inducing inflammatory responses and immunosuppression, and increasing the susceptibility to secondary

* Corresponding author.
E-mail address: simon.mackenzie@stir.ac.uk (S. MacKenzie).

https://doi.org/10.1016/j.aquaculture.2023.740455
Received 7 July 2023; Received in revised form 1 December 2023; Accepted 4 December 2023
Available online 13 December 2023

infections (Fast et al., 2007; Llewellyn et al., 2017; McCarthy et al., 2012; Novak et al., 2016; Oelckers et al., 2014). Consequently, mortalities may occur in heavily infested fish due to osmoregulatory dysfunction and secondary infections.

Salmon lice control is crucial not only to decrease production losses and improve farmed salmon health and welfare but also to protect the surrounding ecosystem and its inhabitants. Several medicinal and non-medicinal treatment strategies have been used to combat lice infestation over the last three decades (as reviewed in Overton et al. (2019)). However, the use of medicinal treatment has dramatically decreased owing to the rapid development of lice resistance and the potential environmental bioaccumulation hazards (Aaen et al., 2015; Burridge et al., 2010). Therefore, alternative non-medicinal strategies have been recently introduced into the salmon industry. Non-medicinal treatment includes four main strategies: cleaner fish, freshwater, mechanical and thermal treatment. Although the use of cleaner fish as a biological control is effective, sustainable, and environmentally friendly, concerns have been raised over their welfare with reports of high mortalities following transfer to salmon cages (Skiftesvik et al., 2014; Treasurer and Feledi, 2014). Freshwater bath treatment using well boats are commonly used to mitigate salmon lice and amoebic gill disease (Hjeltnes et al., 2018), however it has been suggested that continued industrial use of freshwater treatment could potentially drive salmon lice towards increased resistance (Ljungfeldt et al., 2017).

Thermal and mechanical delousing offers a promising environmentally friendly technology for salmon lice treatment. Mechanical treatment includes three different commercially available systems: Hydrolicer, Flatsetsund (FLS) Engineering AS, and SkaMik AS. In such systems, fish are pumped into the treatment system and lice are mechanically removed by flushing using low-pressure washers or vacuuming with inverse turbulence (Gismervik et al., 2017; Hjeltnes et al., 2018; Overton et al., 2019). Although delousing efficiency has been reported, mechanical methods have the potential to induce scale loss, gill bleeding and injuries, and mortalities (Hjeltnes et al., 2018; Overton et al., 2019). Thermal delousing offers an alternative to mechanical delousing and usage has dramatically increased since 2015 such that it accounts for >60% of all lice treatment used in the salmon industry (Overton et al., 2019). Thermal delousing relies on thermal inactivation and detachment of salmon lice after a short exposure to warm water. This treatment is possible from a host perspective as salmonids can tolerate rapid transitory exposure to temperatures of 30–34 °C (Elliott and Elliott, 1995). The lice upper-temperature limit is similar, however the size differential between salmon and lice results in a shorter survival time for lice at suboptimal temperatures (Grøntvedt et al., 2015). Currently, two commercial thermal delousers are available to the industry, Thermolicer® and Optilicer®. Both systems take salmon from the cages into temperate seawater at 28–34 °C, adjusted according to sea temperature and delousing effect, for a short period of time of 20–30s. The main difference between the two technologies is the continuous flow-through system of the Thermolicer® that treats salmon directly pumped from net pens through pipes with heated seawater, whereas Optilicer® uses paddle wheels to push the fish at a pre-set speed through a tank with warm water. Reported efficiencies for Thermolicer® are 75–100% removal of mobile lice stages, and 98% removal of mobile lice stages for Optilicer® (Grøntvedt et al., 2015; Roth, 2016). Nevertheless, neither of the two systems is effective in removing attached lice.

Although thermal treatment has become one of the major delousing techniques, little is known about its impact on salmon health and welfare and the broad-scale effect of post-treatment outcomes. Nilsson et al. (2019) reported that Atlantic salmon exposed to temperatures exceeding 28 °C for 5 mins showed instant behavioural responses indicative of pain. Gismervik et al. (2019) reported Atlantic salmon exposed to 34–38 °C for 72–140 s had associated injuries to gills, eyes, and brain in a pilot laboratory trial however these temperatures are well beyond those used for thermal treatments. Several studies have recorded mortalities and lesions including gill haemorrhage and hyperplasia, skin and scale losses, brain haemorrhages, and affected nasal epithelial and thymus tissue (Østevik et al., 2022; Poppe et al., 2018; Sviland Walde et al., 2021). It is not clear however, whether such pathological features are due to thermal injury, or other treatment-associated factors such as fish pumping and crowding.

The mucosal surface of the gills constitutes the most extensive biological interface between the fish and the external environment. This surface is colonised by a complex highly diverse microbial community that promotes gill mucosal homeostasis and thereby contributes to overall fish health (Elsheshtawy et al., 2021). Concerns have been raised about the impact of thermal treatment on gill health, due to the extremely delicate structure of the organ (Strzyżewska et al., 2016). Recently, an increased prevalence of the gill pathogen *Candidatus Branchiomonas cysticola* and changes in mRNA abundance of cellular stress, inflammation, repair, and proliferation genes in gill tissue were reported after thermal delousing (Østevik et al., 2022). The potential impacts of thermal delousing and associated stressors on the gill microbiome are still unknown. Therefore, this study aimed to provide a detailed understanding of the impact of thermal delousing on farmed Atlantic salmon gill health through (1) exploring the perturbations of salmon gill microbial community after thermal delousing in aquaculture conditions; (2) assessing the histopathological changes and the prevalence of gill pathogens after thermal delousing; and (3) evaluating the impact of thermal delousing on the mRNA abundance of key markers of salmon gill immune response.

2. Materials and methods

2.1. Ethics statement

This study was carried out in accordance with the UK Animal Scientific Procedures Act. The study protocol was approved by the University of Stirling Animal Welfare and Ethical Review Body (AWERB (19/20) 63) and the Norwegian Animal Research Authority (NARA) in 2019 under the identification code 18259.

2.2. Study sites and thermal delousing

Thermal delousing field Trials were conducted at two Atlantic salmon production sites located in Hordaland, Western Norway, with distinct husbandry practices and health conditions. The first site comprised six PE-rings, floating flexible cages with a circular floater (polyethylene) pipes and permeable nets. A total of 140,700 smolts (StofnFiskur strain) with an average weight of 153 g were transferred to the PE-ring included in this study in September 2018. The fish had not undergone any previous treatments and were not diagnosed with any diseases prior to the Trial. The average daily mortality rate in the PE-ring included in this study in the month preceding the Trial was 9 fish. Before the Trial, the average fish weight was 1050 g, the sea temperature was 6 °C and the salinity was recorded at 32%. Thermal delousing Trial 1 was carried out at this site in April 2019 (week 14) using a treatment boat equipped with a Thermolicer. Crowding was conducted by raising the net wall in gradual steps. The treatment involved applying a temperature of 34 °C for 30 s.

The second site consisted of six PE-rings. A total of 162,000 smolts (SalmoBreed strain) with an average weight of 78 g were transferred to the PE-ring included in this study in September 2018. In contrast to the first site, the fish at this location had received four previous treatments: freshwater treatment in April 2019 (week 16), Thermolicer (week 29), Optilicer (week 31), and mechanical delousing using Skamik (week 33). These fish were diagnosed with cardiac myopathy syndrome (CMS) and gill disease (100% prevalence of the gill pathogens *Paranucleospora theridion, Paramoeba perurans, Ichthyobodo salmonis*, *Candidatus Branchiomonas cysticola* and *Cand. Piscichlamydia salmonis*). The average daily mortality rate in the PE-ring included in this study in the month preceding the Trial was 110 fish. Prior to the Trial at this site, the

average fish weight was 2900 g, the sea temperature was 14.2 °C, and the salinity was measured at 32%. Thermal delousing Trial 2 was conducted at the second site in week 34 using Thermolicer. The treatment involved exposing the fish to a temperature of 34 °C for 30s.

2.3. Sampling

Atlantic salmon samples were collected from the two sites: site 1 (n = 60, 30 before and 30 after, 1046 ± 188 g, 45 ± 3 cm) and site 2 (n = 60, 2894 ± 502 g, 61 ± 4 cm), before and after the thermal treatment. Additionally, during the treatment process at each site, 30 samples were collected from the fish that died during treatment. The sampling before and after treatment was carried out on-site, whereas the fish that died during treatment were transported to the research laboratory (FDRG) at the University of Bergen for further sampling. Due to practical field limitations, sampling was conducted 11 and 2 days before treatment and 3 and 7 days after treatment at Sites 1 and 2 respectively. The fish were euthanized using Benzoak vet (ACD Pharmaceuticals AS). The weight, length, preadult and adult lice count, skin ulcers, gill pathology, and changes in the viscera were recorded for each specimen. Gill swabs were collected from the second right gill arch and stored in Longmire's buffer (Longmire et al., 1997) for microbiome analysis. For histological examination, the second gill arch on the left side was collected and fixed in 10% buffered formalin. The third gill arch on the right side was frozen in separate tube on dry ice and stored at −70 °C for real-time RT PCR analyses for the presence of known salmon pathogens. Gill tissue samples from the third left gill arch were collected and preserved in RNA later for subsequent analysis of immune gene expression.

2.4. Microbiome analysis

Gill microbiome analysis was performed as described by Elsheshtawy et al. (2021). In brief, DNA was extracted from the samples using E.Z.N.A.® Tissue DNA Kit (Omega Bio-Tek Inc., USA) according to manufacturing protocol with some modifications. The modifications involved pre-lysis heating of the samples to 95 °C for 10 min to enhance the efficiency of DNA extraction from gram-positive bacteria and utilising Longmire's buffer as a lysis buffer. DNA was finally eluted using 100 μL elution buffer. The DNA purity and concentration were evaluated using a Nanodrop ND-1000 Spectrophotometer (ThermoFisher Scientific, UK) and concentrations were confirmed using a Qubit 2.0 Fluorometer (ThermoFisher Scientific, UK). The bacterial 16S rRNA gene copy numbers were quantified in the samples using absolute qPCR assay as described by Clokie et al. (2022). The primers and probe used for microbiome analysis are listed in Supplementary Table S1. qPCR was performed for all samples in triplicate using SensiFAST Probe Lo-ROX Mix (Bioline, UK). All qPCR runs showed good linearity (R^2 = 0.993–1, $p < 0.05$) and amplification efficiency of 95–101%. Template DNA used to build the amplicon libraries was normalized to an equal 16S rRNA concentration (1e6 copies) according to the qPCR assay results. Bacterial 16S rRNA Illumina amplicon libraries were generated using a two-step PCR amplicon assay from all the samples, negative sequencing control (NSC), no template control (NTC), and a positive microbiome control. The V4 region of the bacterial 16S rRNA gene was PCR-amplified using primers over hanged with Illumina adaptors. The first PCR cycling conditions were as follows: initial denaturation at 98 °C for 2 min, 25 cycles of denaturation at 98 °C for 15 s, annealing at 54 °C for 30 s, and extension at 65 °C for 45 s, followed by a final extension step at 65 °C for 10 min. Purified first PCR products were barcoded by the addition of unique index sequences to the 5′ and 3′ ends of each sample using Nextera XT Index Kit (Illumina, USA). The second PCR was performed with the same conditions as the first PCR for 8 cycles. Purified second PCR amplicons were quantified using Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, USA) following the manufacturer's protocol. An equimolar final pool was prepared from the samples and sequencing was performed by Novogene (Cambridge, United Kingdom) at PE250 using an S4 flowcell on an Illumina Novaseq (Illumina, United States).

The raw sequence data provided by Novogene contained 128 paired fastq files. Sample sequence data (fastq files) were processed following Mothur's SOP (Schloss et al., 2009) and the SILVA 132 reference database (Quast et al., 2013). The total number of the retrieved raw reads was approximately 61.3 million and the number of sequences per sample ranged between 148,131 and 1,311,471 with an average of 502,748 reads. All statistical analysis was performed in R studio (Version 1.2.5042). Alpha diversity was calculated using Phyloseq package (McMurdie and Holmes, 2013). Alpha diversity of gill bacterial community was estimated using richness (Chao1 and Ace indices) taxa abundance and evenness (Shannon-Weaver and inverse Simpson). The homogeneity of variance of the alpha diversity indices was tested using Shapiro–Wilks test before testing the differences between groups. Alpha diversity metrics were analysed using t-test when the data were normally distributed, whereas the Wilcoxon test (rank sum test) was used for non-normally distributed data, and p-values were adjusted using Benjamini and Hochberg (BH) correction (Benjamini and Hochberg, 1995). All statistical analysis was conducted with the rstatix package (Kassambara, 2020b). Beta diversity comparisons were calculated using Bray-Curtis pairwise distances in packages vegan (Oksanen et al., 2013) and phyloseq and visualised using non-metric multidimensional scaling (NMDS). Differences between groups were calculated using non-parametric permutational multivariate analysis of variance (PERMANOVA) of 1002 permutations with vegan package. Differences between groups were considered statistically significant at adjusted $p < 0.05$. All figures were produced using the R package ggpubr and ggplot2 (Kassambara, 2020a; Wickham, 2016). In order to compare the relative abundance of taxa between different groups, we generated differential heat trees using the Metacoder R package (Foster et al., 2017). The trees illustrate the log2 fold change in taxa abundance. A Wilcoxon rank-sum test followed by a Benjamini-Hochberg (FDR) correction was applied to test the differences between the same taxa in the two timepoints and p-value was set to 0.05. In addition, the significant genera between the two time points were identified using the Wilcoxon rank-sum test followed by a Benjamini-Hochberg (FDR) correction and the p-value was set to 0.05.

2.5. Quantification of salmon pathogens in gill samples

2.5.1. RNA extraction

RNA was extracted from gill samples using a standard protocol for TRIzol® Reagent (Life Technologies, US) as described by Gunnarsson et al. (2017). A negative extraction control was included for every 10 samples. Tissues were homogenized using a TissueLyser II (Qiagen, Germany) for 3 min at a speed of 30 Hz. The RNA pellets were washed twice using 1 ml 75% ethanol. The RNA pellets were eluted in 100 μL RNase-free water. RNA concentrations (ng/μL) were measured for each sample using a NanoDrop™ 1000 (ThermoFisher Scientific, UK), and the RNA samples were stored at −70 °C until further analyses.

2.5.2. Real-time PCR (qPCR)

Real-time RT-PCR analyses of a selection of the main pathogens associated with diseases in farmed Atlantic salmon were performed using AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems, US). qPCR assays included *Isavirus salaris* (ISAV-7), *Salmon gillpox virus* (SGPV-MCP), *Salmon pancreas disease virus* (SAV-nsp1), *Piscine myocarditis virus* (PMCV), *Piscine orthoreovirus* (PRV1-M2), *Aquabirnavirus salmonidae* (IPNV), *Candidatus Branchiomonas cysticola* (Ca. Bcyst), *Candidatus Piscichlamydia salmonis* (Ca. Psal), *Candidatus Syngnamydia salmonis* (Ca. Ssal), *Paranucleospora theridion* (Pther), *Paramoeba perurans* (Pperu), *Parvicapsula pseudobranchicola* (Parvi), and *Ichthyobodo* spp. (Costia). The elongation factor 1 alpha (EF1A) was used as a reference gene. Primers and probes used in this study are listed in Supplementary Table S1.

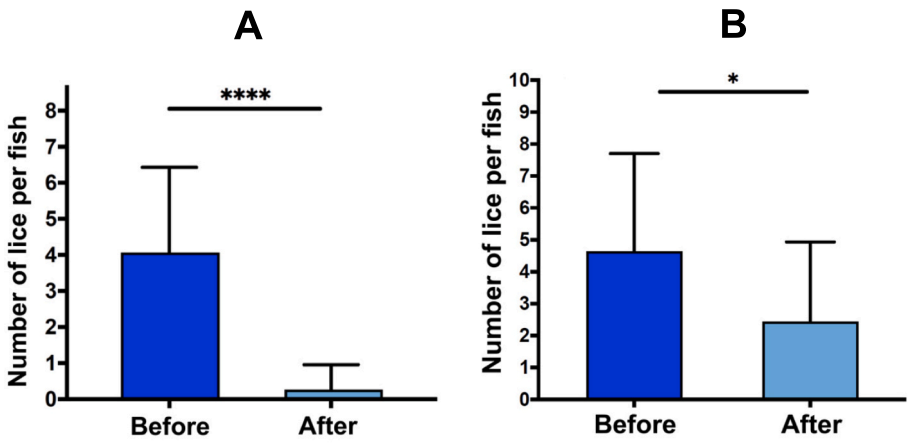


Fig. 1. Mean count of mobile lice (preadult and adult) on Atlantic salmon before and after thermal delousing in Trials 1 (A) and 2 (B). Data are presented as mean ± SD ($n = 30$), and *$p < 0.05$, and **** $p < 0.0001$.

2.6. Statistical analysis

The density of the target pathogens is expressed as Normalized Expression (NE). qPCR Efficiencies (E) and Ct-values were used to calculate the normalized expression of the targets using the EF1A as a reference gene: $NE = (E_{EF1A})^{CT\ EF1A} / (E_{target})^{CT\ target}$. Density data were tested for normality using Shapiro-Wilks test. Kruskal-Wallis, a non-parametric test, was used to test the difference in pathogen density in samples collected before, during and after the treatment. The Kruskal-Wallis test was followed by Dunn's multiple comparison test. A p-value <0.05 was considered significant in all analyses (* $= p \leq 0.05$, ** $= p \leq 0.01$, *** $= p \leq 0.001$, **** $= p \leq 0.0001$). The prevalence is given as the percentage of positive individuals: Prevalence (%) = Number of positive samples /total number of tested samples x 100.

2.7. Histopathology

The formalin-fixed tissues were embedded in paraffin and sectioned at IDEXX (Wetherby, UK). The 2 μm sections were mounted on poly-L-lysin coated slides (SuperfrostPlus, Thermo Scientific) and stained with haematoxylin-eosin (HE) using a standard method (Culling et al., 1985). The histological scoring of the stained sections was carried out at the University of Bergen. A scoring system based on 11 known pathological changes in the gills was used; I) Mucus cell hyperplasia, II) Clubbing, III) Lifting, IV) Epithelial cell hypertrophy, V) Epithelial cell hyperplasia, VI) Thickening of the distal primary lamella, VII) Fresh aneurism, VIII) Bleeding aneurism, IX) Old aneurism, X) Inflammation, and XI) Necrosis. The following scores were given for each character: A) 0 = no changes observed, B) 1 = <10% of the tissue was affected, C) 2 = between 10 and 50% of the tissues were affected, and D) 3 = >50% of the tissue were affected.

2.8. Immune gene expression analysis

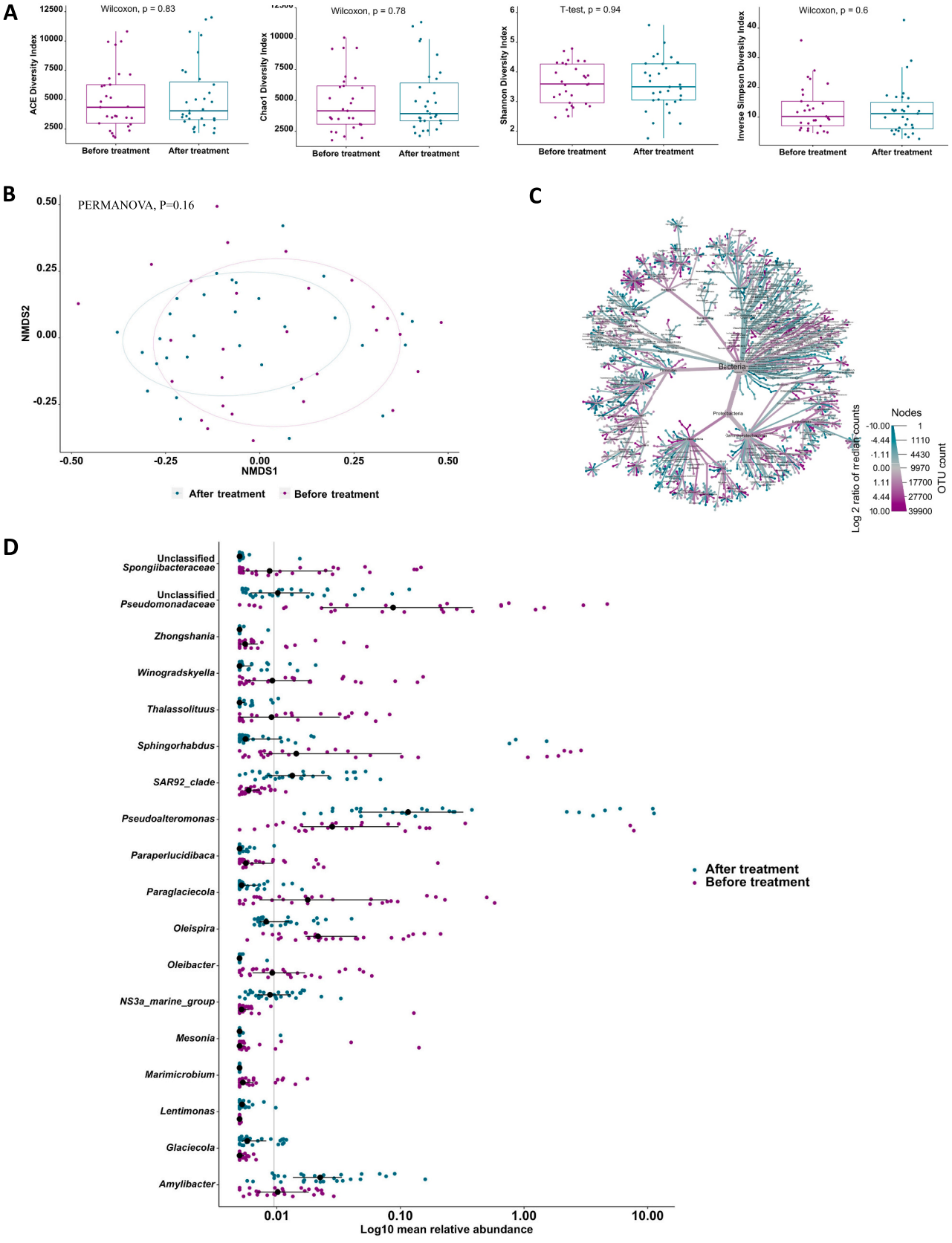
Total RNA was extracted from a 100 mg of gill tissue samples using TRI Reagent (Sigma-Aldrich, USA) following to the manufacturer's protocol. The extracted RNA was eluted in 100 μL DEPC-treated Water (ThermoFisher Scientific, UK). The quality and quantity of RNA samples were evaluated using a Nanodrop ND-1000 Spectrophotometer (ThermoFisher Scientific, UK). The integrity of the RNA was evaluated by 1.5% (v/v) agarose gel (Sigma-Aldrich, USA) containing 0.1 μg/mL ethidium bromide (Sigma-Aldrich, USA) in Tris-Acetate-EDTA (TAE) buffer (Sigma-Aldrich, USA). cDNA was synthesised and genomic DNA contamination was removed from 1 μg of the purified RNA sample using QuantiTect® Reverse Transcription (Qiagen, Germany) according to the manufacturer's protocol. To confirm successful reverse transcription, the cDNA was tested using PCR for *Salmo salar β -actin* gene. A real-time absolute qPCR was performed to quantify the target genes copy numbers in the samples. Primers were designed for the following Atlantic salmon genes *mhc ii*, *il-1β*, *tnf-α*, *il-10* and *tgf-β* by using NCBI Primer design tool (Supplementary Table S1). The designed primers were optimised, and the clean PCR products were ligated into a vector using pGEM®-T Easy Vector Systems (Promega, UK) and transformed into XL1-Blue Competent Cells (Agilent Technologies, USA) following the manufacturer's protocol. The plasmid DNA was extracted using NucleoSpin Plasmid Quick Pure (Macherey-Nagel, Germany). Successful cloning was confirmed using PCR and plasmid DNA Sanger sequencing using T7 promoter (GTAATACGACTCACTATAGGGC) (Eurofins Genomics, UK). The sequence was aligned with the specific primers for the insert using Clustal Omega Multiple Sequence Alignment software and the obtained product sequence was blasted in the gene bank to check the correct insertion. Then, copy numbers per μL of plasmid DNA were calculated and used for generating a standard curve for absolute quantification. qPCR was performed for all samples in triplicate using Luminaris Colour HiGreen qPCR Master Mix (ThermoFisher Scientific, UK) in Stratagene MX3005p qPCR (Agilent Technologies, USA). All qPCR runs showed good linearity ($R^2 = 0.996–1$, $p < 0.05$) and amplification efficiency of 97–102%.

Immune gene expression data were analysed using GraphPad Prism version 8.4.2 (San Diego, CA, USA). The obtained data were tested for normality by assessing the frequency of distribution in the histogram and using Anderson-Darling test, D'Agostino & Pearson test, Shapiro-Wilk test, and Kolmogorov-Smirnov test. All results are reported as means and standard deviation (SD). Normally distributed data were tested using independent *t*-test, whereas non-parametric data were tested by Wilcoxon test. Differences between groups were considered statistically significant at $p < 0.05$.

3. Results

3.1. Impact of thermal delousing on sea lice count and farm mortalities

Both delousing trials showed a significant reduction in the number of mobile life stages of sea lice (Fig. 1). The average loads of L. *salmonis* mobile stages (pre- and adult lice) were 4.1 and 4.6 before treatment, which significantly decreased post-treatment to 0.3 ($p < 0.001$) and 2.4 ($p < 0.05$) at sites 1 and 2, respectively. Chalimi stages were not included in the counts for both trials. However, it is important to note that the sea temperature at Site 2 was 15 °C, indicating that the attached chalimi stages could have moulted into pre-adult stages by the time of



(caption on next page)

Fig. 2. Impact of thermal treatment (Sea lice thermal treatment Trial 1) on the Atlantic salmon gill microbiome ($n = 30$). (A) Alpha diversity metrics of salmon gill microbial communities before and after treatment. Dots represent each individual sample. (B) Non-metric multidimensional scaling (NMDS) plots based on Bray–Curtis similarity matrix of gill microbial communities before and after treatment (PERMANOVA, $p = 0.16$). The colours of the ellipses represent the two groups. (C) Metacoder heat-tree showing the difference in gill microbiome phylotypes before and after treatment. Nodes in the heat-tree correspond to phylotypes, as indicated by node labels, while edges link phylotypes in accordance with the taxonomic hierarchy. Node sizes correspond to the number of observed OTUs. Colours represent the log fold difference of a given phylotype's median relative abundance pre-treatment compared to post-treatment. Only significant differences, Wilcox rank-sum test followed by a Benjamini-Hochberg (FDR) correction are coloured. Taxa coloured dark cyan represent enrichment after treatment and dark magenta before treatment. (D) Significant different genera in Atlantic salmon gill microbiome (n = 30) upon thermal treatment (sea lice Trial 1). Only significantly different genera between before and after treatment at $p < 0.05$ are presented, Wilcox rank-sum test followed by a Benjamini-Hochberg (FDR) correction. Coloured dots represent log10 mean relative abundance for each individual sample and black circles indicate the median and the black lines indicate the 50% confidence intervals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sampling, 7 days after treatment.

Fish mortalities increased at both sites during the thermal delousing trials. In Trial 1, the mortality rate increased from an initial level of 0.006%/day before treatment to 0.4%/day on the following four days after treatment. However, after a few days, the mortality rate returned to the same level as before treatment. No skin lesions were observed on the dead fish or in samples collected after treatment. In Trial 2, the mortality rate increased from 0.07%/day before treatment to 0.8%/day on the treatment day, followed by a drop to daily mortality of around 0.1%/day a few days after treatment. The daily mortality rate levelled off at 0.02%/day in the following 23 days. Samples collected both before and after treatment showed the presence of skin ulcers, bleeding, and skin damage caused by sea lice. All the fish dying during treatment exhibited skin haemorrhages, and 26 out of the 30 dead fish, had blood in the pericardial cavity.

3.2. Impact of thermal delousing on Atlantic salmon gill microbiome

To explore the impact of thermal treatment on Atlantic salmon gill microbial communities, we compared alpha and beta diversity before and after thermal treatment and further identified the taxonomic variations between the two timepoints. In the first thermal Trial (Site 1), no significant differences were observed in alpha diversity indices for richness (Chao1 and ACE) and evenness (Shannon-Weaver and Inverse Simpson) (Wilcoxon and *t*-test, $p > 0.05$; Fig. 2A). PERMANOVA analysis of dissimilarity demonstrated no significant difference across the gill microbial communities before and after thermal treatment at site 1 (PERMANOVA, $p = 0.16$, $R^2 = 0.02341$; Fig. 2B), suggesting that the community composition had not significantly changed after treatment. The differential abundant taxonomies before and after treatment are presented in a heat tree (Fig. 2C). Among the two time points, only 18 bacterial genera were identified as significantly different (Wilcoxon, $p < 0.05$; Supplementary Table S2, Fig. 2D).

In contrast, all measured alpha indices were significantly different upon thermal treatment in Trial 2 (Site 2) (Wilcoxon, $p < 0.000001$; Fig. 3A). Gill microbial richness and evenness were remarkably higher after thermal treatment Trial 2. Moreover, beta diversity among groups also showed that samples clustered according to the timepoint, indicating that the salmon gill microbiome had a distinct compositional profile pre- and post-thermal treatment (PERMANOVA, $p < 0.001$, $R^2 = 0.32076$; Fig. 3B). The heat tree shown in Fig. 3C demonstrated that several bacterial taxonomies are considerably enriched after treatment. We have further identified 99 differentially abundant genera between the two timepoints (Wilcoxon, $p < 0.05$; Supplementary Table S3). Of these, 37 were highlighted as highly significant different genera (Wilcoxon, $p < 0.01$; Fig. 3D).

3.3. Impact of thermal delousing on the prevalence and density of gill pathogens

The effect of thermal treatment on the prevalence of microparasites, as assessed through real-time RT PCR, was limited (Table 1). In Trial 1, there was a minor increase in the prevalence of *P. theridion* and *Ichthyobodo salmonis* (costia assay), accompanied by a slight drop in the prevalence of *P. pseudobranchicola*. Similarly, Trial 2 exhibited comparable findings, revealing a reduction in the prevalence of SGP virus, *Cand. S. salmonis*, and *P. pseudobranchicola*, alongside an increase in the prevalence of PMCV.

Likewise, minor changes in pathogen density were observed after thermal delousing. In Trial 1, there was a significant increase in the density of *Cand. B. cysticola* ($p < 0.0001$), and *P. theridion* ($p < 0.05$), along with a reduction in the density of PRV1 after treatment ($p < 0.0001$). In the second Trial, a notable increase in the density of *Cand. P. salmonis* and *P. theridion* was observed ($p < 0.01$), whereas the density of *Ichthyobodo* sp. exhibited a significant decrease after treatment ($p < 0.01$). No other significant changes in pathogen density were observed for the other microparasites in either of the Trials following treatment.

3.4. Histopathological and immunological status of the gills in response to thermal delousing

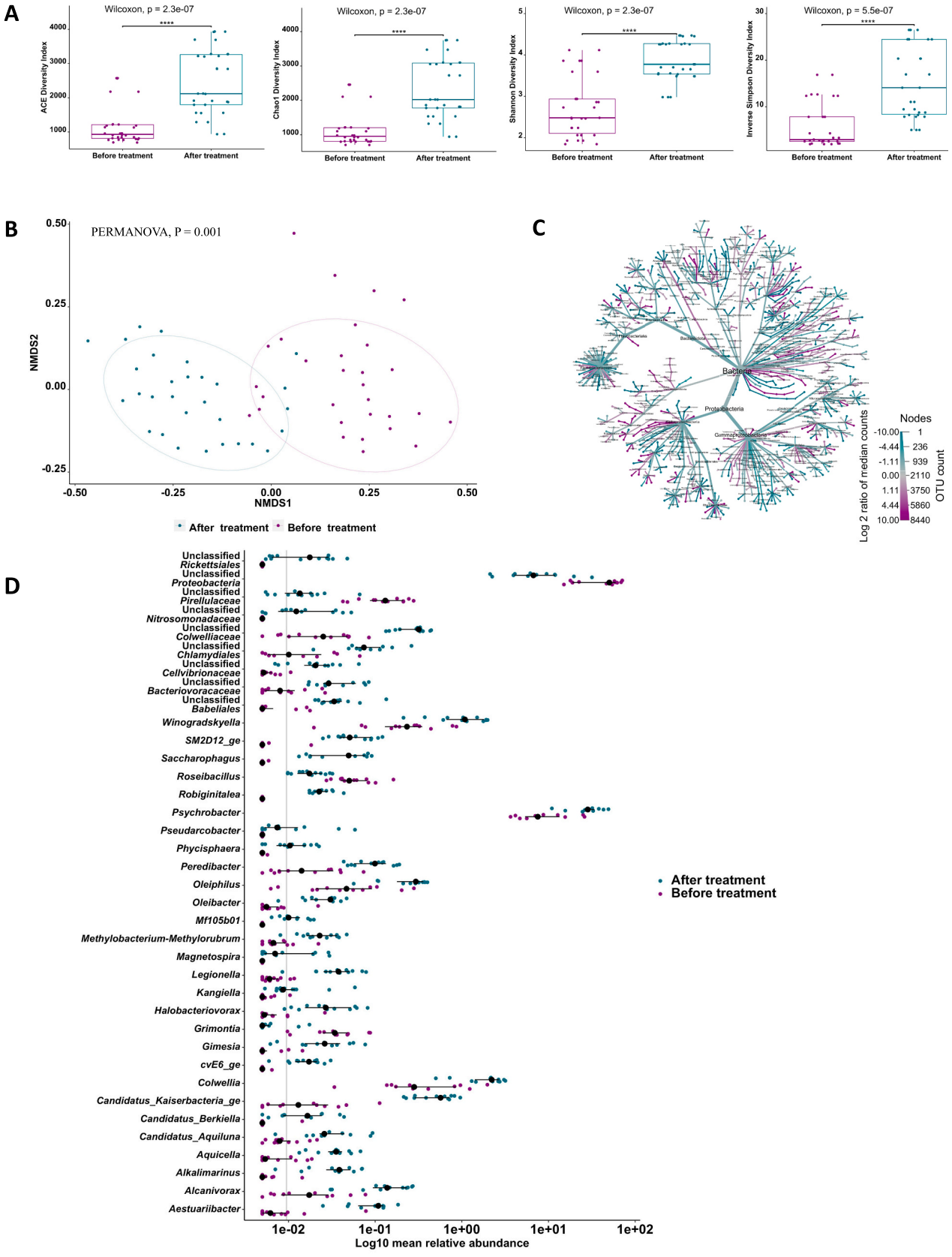
In Trial 1, prior to treatment, the histological gill score showed mild changes. Nevertheless, the treatment did not lead to any significant increase in gill pathology, as depicted in Fig. 4A. The most notable gill score before treatment was observed for clubbing of the secondary lamellae, which subsequently decreased following treatment. Additionally, the average score for mucus cell hyperplasia also decreased after treatment. Nonetheless, there was a slight increase in gill score post-treatment for epithelial cell hypertrophy and hyperplasia, thickening of the distal filament, old aneurism, and inflammation, as illustrated in Fig. 4. In Trial 2, the average histological gill scores before treatment were slightly high compared to Trial 1 (Fig. 5A). The highest gill scores before treatment were associated with mucus cell hyperplasia, epithelial cell hypertrophy and hyperplasia, and aneurysms. Following treatment, there was a general decrease in the histological gill score, with the exception of a slight increase observed in clubbing and the occurrence of fresh and old aneurysms (Fig. 5).

To evaluate the impact of the thermal delousing on key markers of the Atlantic salmon gill immune response, we performed absolute qPCR gene expression analysis for antigen-presenting cells (*mhc ii*), proinflammatory cytokines (*il-1β* and *tnf-α*) and inhibitory cytokines (*tgf-β* and *il-10*). The abundance of mRNA transcripts of all tested targets was downregulated after thermal treatment of sea lice in both Trials (*t*-test, $p < 0.05$; Fig. 6), indicating that thermal treatment caused a suppression of the measured mRNAs.

4. Discussion

4.1. Effects of thermal delousing on sea lice count and treatment-associated mortalities

The efficacy of thermal delousing in removing a significant majority of the preadult and adult stages of L. *salmonis* in Atlantic salmon has been well-documented (Grøntvedt et al., 2015; Nilsson et al., 2023; Roth, 2016). The present study also confirmed these findings, showing a notable reduction in the prevalence of these stages following treatment. Of note, thermal delousing has little effect on the attached chalimus stages (Andrews and Horsberg, 2021). Thus, the reduced effectiveness



(caption on next page)

Fig. 3. Impact of thermal treatment (Sea lice thermal treatment Trial 2) on the Atlantic salmon gill microbiome (n = 30). (A) Alpha diversity metrics of salmon gill microbial communities before and after treatment. Dots represent each individual sample and ****$p < 0.0001$. (B) Non-metric multidimensional scaling (NMDS) plots based on Bray–Curtis similarity matrix of gill microbial communities before and after treatment (PERMANOVA, $p = 0.001$). The colours of the ellipses represent the two groups. (C) Metacoder heat-tree showing the difference in gill microbiome phylotypes before and after treatment. Nodes in the heat-tree correspond to phylotypes, as indicated by node labels, while edges link phylotypes in accordance with the taxonomic hierarchy. Node sizes correspond to the number of observed OTUs. Colours represent the log fold difference of a given phylotype's median relative abundance pre-treatment compared to post-treatment. Only significant differences, Wilcox rank-sum test followed by a Benjamini-Hochberg (FDR) correction are coloured. Taxa coloured dark cyan represent enrichment after treatment and dark magenta before treatment. (D) Highly significant different genera in Atlantic salmon gill microbiome (n = 30) upon thermal treatment in sea lice Trial 2. Only significantly different genera between before and after treatment at $p < 0.01$ are presented, Wilcox rank-sum test followed by a Benjamini-Hochberg (FDR) correction. Coloured dots represent log10 mean relative abundance for each individual sample and black circles indicate the median and the black lines indicate the 50% confidence intervals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Prevalence of selected pathogens in Atlantic salmon gill tissue samples collected before and after thermal delousing at two commercial sites.

Pathogen	Trial 1				Trial 2			
	Before treatment		After treatment		Before treatment		After treatment	
	Number of positive samples	Prevalence	Number of positive samples	Prevalence	Number of positive samples	Prevalence	Number of positive samples	Prevalence
ISAV-7	12	40%	12	40%	3	10%	5	16,7%
SGPV-MCP	0	0%	0	0%	8	26.7%	1	3.3%
SAV3	0	0%	0	0%	0	0%	0	0%
PRV1-M2	30	100%	30	100%	30	100%	30	100%
PMCV	0	0%	0	0%	21	70%	27	90%
IPNV	0	0%	0	0%	0	0%	0	0%
Cand. B. cysticola	30	100%	30	100%	30	100%	30	100%
Cand. P. salmonis	0	0%	0	0%	30	100%	30	100%
Cand. S. salmonis	0	0%	0	0%	30	100%	28	93,3%
P. theridion	29	96,7%	30	100%	30	100%	30	100%
P. perurans	0	0%	0	0%	30	100%	30	100%
P. pseudobranchicola	3	10%	1	3.3%	18	60%	6	20%
Ichthyobodo sp.	0	0%	4	13,3%	30	100%	30	100%

observed in Trial 2, after treatment at a sea temperature of 14.1 °C, could be attributed to the moulting of chalimus stages into preadult stages (Hamre et al., 2019). In addition, the detached lice are collected following delousing, although the effectiveness of this process may vary. Consequently, there is a potential for some of the lice that are initially removed during treatment to reattach to salmon in neighbouring pens. It is noteworthy that both farms included in this study had six polar net pens.

Thermal delousing has been linked to detrimental effects on fish health and welfare including external damage to the skin and gills, increased density of gill pathogens, and elevated mortalities (Bui et al., 2022; Gismervik et al., 2019; Moltumyr et al., 2022; Østevik et al., 2022; Overton et al., 2019). In the present study, observations during Trial 1 revealed a minimal increase in mortality during treatment, which subsequently returned to the baseline levels of daily mortality observed prior to the treatment. Trial 2 demonstrated a rise in mortality from 0.07% to 0.8% during treatment, followed by a decrease to 0.02% over the subsequent 23 days. Notably, no significant increase in mortality was observed during the post-treatment observation period. High mortalities observed in Trial 2 can be possibly linked to the coexistence of Cardiomyopathy Syndrome (CMS) and gill disease, suggesting that factors (health status and husbandry history) beyond the thermal treatment itself may influence mortality rates in the studied population.

4.2. Impact of thermal delousing on Atlantic salmon gill microbiome

The impact of thermal treatment was examined at two different study locations, with different husbandry and health backgrounds. Our results from Site 1 showed no significant differences in the gill microbial community, whereas findings from Site 2 demonstrated a significant impact, consisting of an increase in salmon gill microbial richness, evenness, and community composition. These findings indicate that thermal treatment had not caused an adverse impact on the microbial communities, which could be attributed to the short-term exposure to a high temperature. Importantly, variations in the gill microbiome in response to treatment between the two sites could be due to the notable differences in the husbandry history and health status of the fish at the two sites. Following a freshwater bath for Amoebic Gill Disease (AGD) in April 2019 (week 16), the fish at Site 2 had received three sea lice treatments (weeks 29, 31 and 33) over the 6 weeks prior to the Thermolicer trial, indicating an ongoing caligidosis issue. In addition, the fish were diagnosed with Cardiomyopathy Syndrome (CMS) and gill inflammation. Whereas fish at Site 1 had not received any treatment and were not diagnosed with any pathologies prior to the Trial. Thus, the gills of the fish at Site 2 may have been adversely impacted before the Trial as a result of multiple husbandry interventions, which had potentially led to gill microbial dysbiosis before the treatment.

Our results demonstrated a significant increase in the gill bacterial community diversity and differing community structure at Site 2 after the treatment. For instance, the genera *Psychrobacter, Photobacterium, Colwellia, Unclassified Colwelliaceae,* and *Psychrobium* abundance significantly increased after treatment in Trial 2. The genus *Psychrobacter* is commonly identified in the skin and gill microbiome, and it has antifungal properties against *Saprolegnia australis* and *Mucor hiemalis* (Bowman, 2006; Lowrey et al., 2015). *Photobacterium* was described among the healthy gill microbiome of Yellowtail Kingfish (*Seriola lalandi*) (Legrand et al., 2018). *Colwellia, Unclassified Colwelliaceae* and *Psychrobium* were described among the essential marine denitrifying bacteria (Lin et al., 2019). In addition, the genera *Bosea, Nitrosomonadaceae Unclassified, Colwelliaceae ge, Ketobacter, Robiginitalea, Bacillales Unclassified, Opitutales Unclassified, Rickettsiales Unclassified, Magnetospira, Parachlamydiaceae Unclassified* and *Novosphingobium* were only detected after treatment. Genera belonging to *Nitrosomonadaceae* and *Colwelliaceae* are crucial for ammonia oxidation. *Bosea* was identified as a core genus of rainbow trout (*Oncorhynchus mykiss*) gill microbiome that produces antagonistic compounds and inhibited the growth of *Flavobacterium psychrophilum* (Takeuchi et al., 2021). Interestingly, these results highlight the potential use of the gill microbiome as a

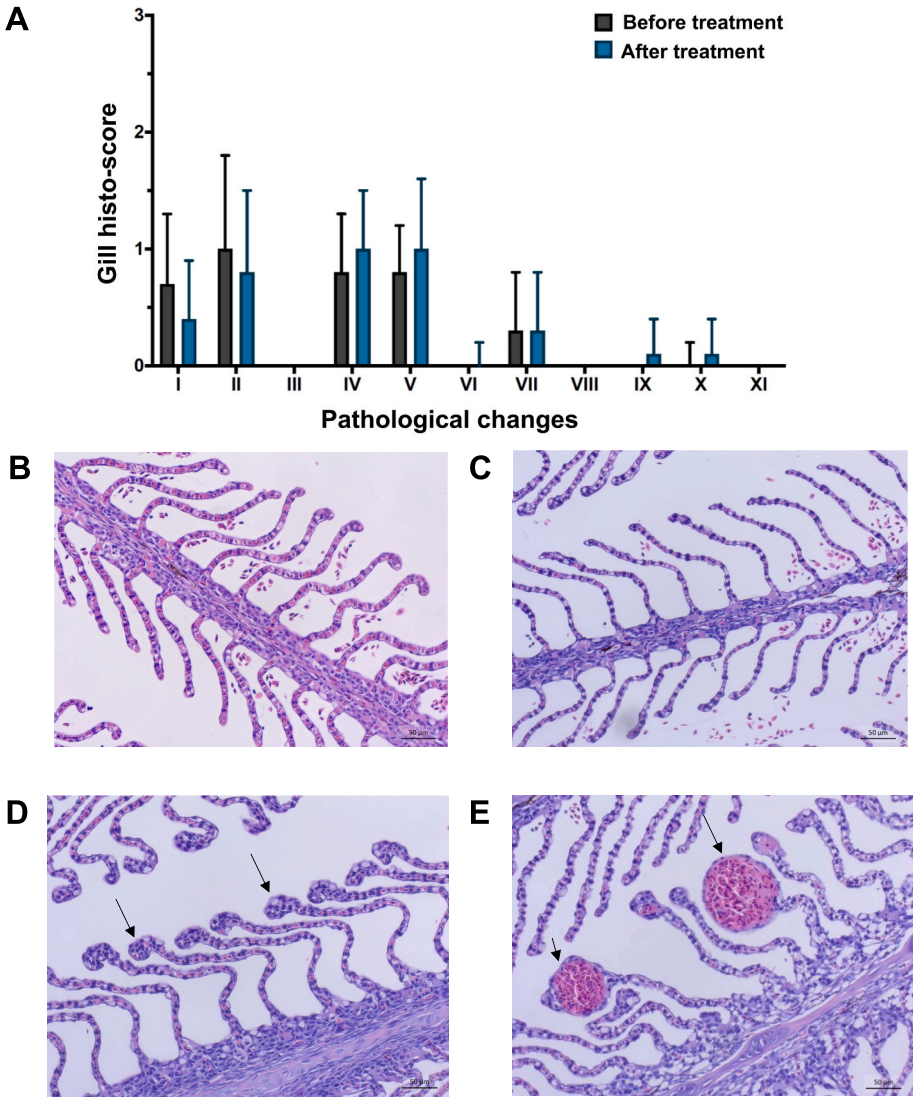


Fig. 4. Histopathological changes in Atlantic salmon gills during thermal delousing Trial 1. (A) Histological score of gill pathology of Trial 1 before and after delousing based on the elven pathological changes: I) Mucus cell hyperplasia, II) Clubbing, III) Lifting, IV) Epithelial cell hypertrophy, V) Epithelial cell hyperplasia, VI) Thickening of the distal primary lamella, VII) Fresh aneurism, VIII) Bleeding aneurism, IX) Old aneurism, X) Inflammation, and XI) Necrosis. The scores were given for each character: 0 = no changes observed, 1 = <10% of the tissue was affected, 2 = between 10 and 50% of the tissues were affected, and 3 = >50% of the tissue were affected. Data are presented as mean ± SD (n = 30). Gill tissue haematoxylin and eosin-stained sections (B) before and (C) after thermal treatment 1. (D) Clubbing and (E) Fresh aneurism observed in gills after treatment.

marker for the health and welfare status of the fish. Changes in the health status of the fish can induce notable variations in gill microbial community structure and diversity such as variations in the microbial richness and evenness, as well as expansions of opportunistic and pathogenic species (Legrand et al., 2018). Our findings indicated also that the impact of thermal treatment of sea lice upon gill microbial communities may differ according to the history of husbandry interventions and their impact upon health status. Our results identify the essential need for longitudinal studies in order to understand the upper and lower limits for the gill microbiome as a proxy for fish health status and to further understand the potential impact on observed variation from location, genetics, seasonality and fish production stage.

4.3. Impact of thermal delousing on the prevalence of gill pathogens

The thermal delousing Trials conducted in this study demonstrated limited impact on the prevalence of the tested pathogens. Nonetheless, significant changes were observed in the density of certain pathogens. In Trial 1, there were notable increases in the densities of *Cand. B. cysticola* and *P. theridion*, while Trial 2 showed significant increases in the densities of *Cand. P. salmonis* and *P. theridion*. These findings align with Østevik et al. (2022), who reported an increase in the load of *Cand. B. cysticola* following thermal delousing. In contrast, there were significant declines in the densities of other pathogens, such as PRV1 in Trial 1 and *Ichthyobodo* sp. in Trial 2. Although the salmon in Trial 2 were infected with PMCV, no substantial increase in PMCV density was observed following the Trial. Based on the findings of the present study and the existing literature, there is limited evidence to suggest that thermal treatments would lead to a significant increase in gill pathogen densities. Given the higher mortalities in diseased salmon subjected to stressors (Garseth et al., 2018), it is recommended that salmon suffering from disease should not undergo thermal treatments (Bui et al., 2022; Garseth et al., 2018; Moltumyr et al., 2022).

4.4. Histopathological and immunological status of the gills in response to thermal delousing

Thermal delousing has been associated with gill pathological

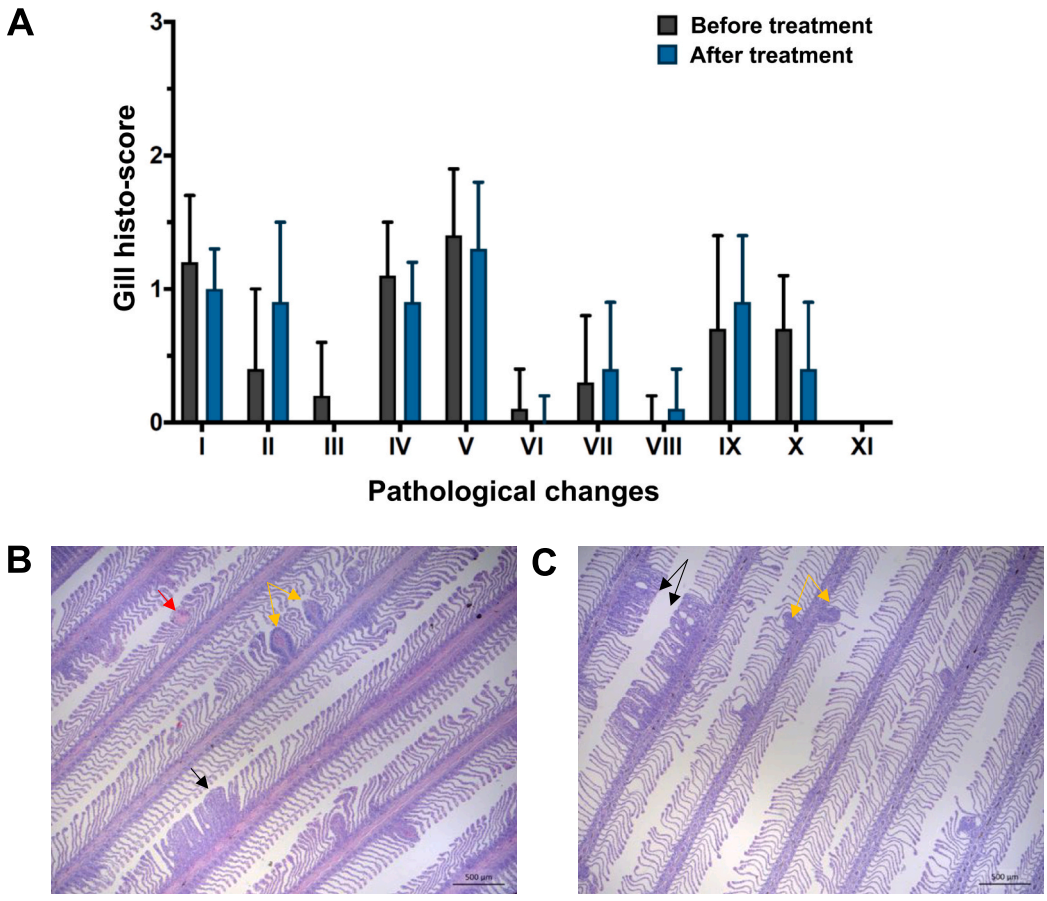


Fig. 5. Histopathological changes in Atlantic salmon gill during thermal delousing Trial 2. (A) Histological score of gill pathology of Trial 2 before and after delousing based on the elven pathological changes: I) Mucus cell hyperplasia, II) Clubbing, III) Lifting, IV) Epithelial cell hypertrophy, V) Epithelial cell hyperplasia, VI) Thickening of the distal primary lamella, VII) Fresh aneurism, VIII) Bleeding aneurism, IX) Old aneurism, X) Inflammation, and XI) Necrosis. The scores were given for each character: 0 = no changes observed, 1 = <10% of the tissue was affected, 2 = between 10 and 50% of the tissues were affected, and 3 = >50% of the tissue were affected. Data are presented as mean ± SD (n = 30). Gill tissue haematoxylin and eosin-stained sections (B) before and (C) after thermal treatment 2, showing epithelial cell hypertrophy and hyperplasia, and adhesions of secondary lamellae (black arrow), old aneurysms (orange arrow), and fresh aneurysms (red arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

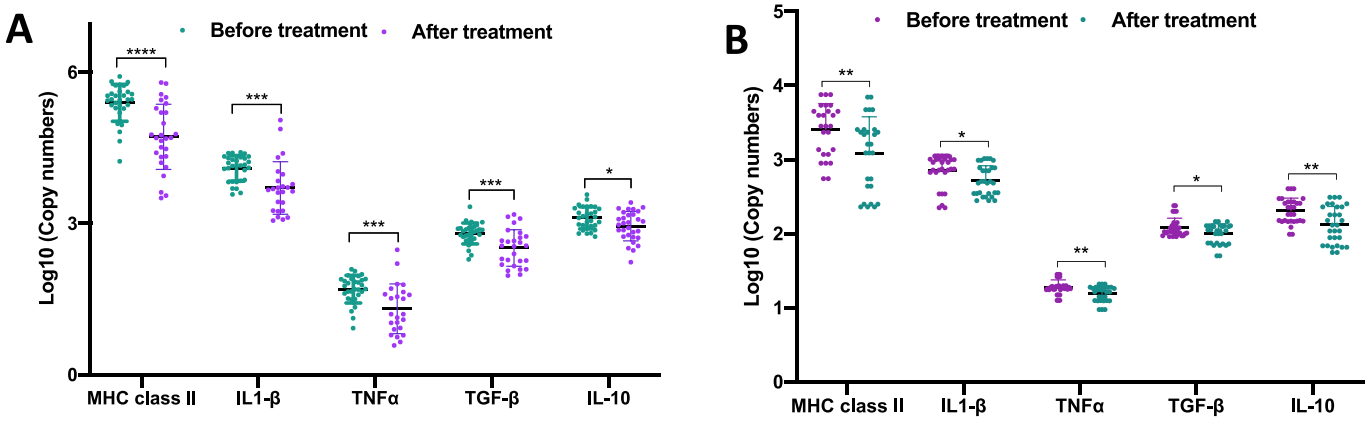


Fig. 6. Impact of thermal treatment of sea lice on the immunological status of the gills, Trial 1 (A) and 2 (B). Data are presented as mean ± SD of log 10 copy numbers of *mhc ii*, *il-1β*, *tnfα*, *tgf-β* and *il-10* at two points (n = 30). Dots represent each individual sample and *$p < 0.05$, ** $p < 0.001$, and *** $p < 0.0001$.

changes, such as aneurysms, gill haemorrhages, and epithelial cell hyperplasia (Gismervik et al., 2019; Grøntvedt et al., 2015; Moltumyr et al., 2021; Moltumyr et al., 2022). In the current study, histological examination of the gills revealed minor changes, including aneurysms and epithelial cells hypertrophy and hyperplasia. Notably, gill bleeding and skin ulcers were observed in samples from Trial 2. There is an ongoing debate regarding delousing associated gill lesions contributing to increased mortalities. Østevik et al. (2022) estimated that <2% of the gill tissues were affected after thermal delousing, and they concluded that the impact of these lesions on increased mortalities was uncertain but could potentially have reduced gill capacity and function. The data from our study emphasise the importance of considering the health

status of the fish prior to treatment. At Site 1, the fish were not diagnosed with any disease, whereas at Site 2, the fish were diagnosed with CMS and gill disease. This disparity in health conditions between the two sites may explain the higher mortalities observed during the second Trial.

On the other hand, both field Trials showed suppression of cytokine mRNAs and *mhc ii* after thermal delousing. Likewise, Rebl et al. (2020) reported reduced bactericidal and inflammatory activity, and a significantly altered blood-cell composition in rainbow trout (*Oncorhynchus mykiss*) upon thermal stress coupled with overstocking. In contrast, Østevik et al. (2022) noted a continued increased mRNA abundance of the pro-inflammatory cytokines *il-1β* and *tnf-α* after thermal delousing, whereas the anti-inflammatory cytokines *il-10* and *tgf-β* were upregulated 1 day post-treatment, and downregulated or plateaued 8 days later. The observed immunosuppression in the current study could be attributed to stressors associated with thermal delousing. In addition to thermal stress, fish are subjected to multiple stressors during delousing such as crowding and pumping that cause acute gill injury that may consequently lead to immune suppression (Gismervik et al., 2019; Nilsson et al., 2019).

In summary, a significant impact of thermal delousing on the gill microbiome can be identified in Atlantic salmon (*Salmo salar*). In this study, the observed effect in one of the commercial populations studied was an increase in gill microbiome diversity whereas at the other commercial study site no differences were observed. Differences observed in immunological activity although significant were minor and pathogen prevalence and density and histopathology were not impacted. The most significant factor observed in this study was the relationship between prior husbandry history and health status of the commercial populations.

CRediT authorship contribution statement

Ahmed Elsheshtawy: Conceptualization, Data curation, Formal analysis, Investigation, Software, Validation, Writing – original draft. **Benjamin Gregory James Clokie:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **Amaya Albalat:** Conceptualization, Investigation, Methodology, Supervision, Validation, Writing – review & editing. **Are Nylund:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Supervision, Validation, Writing – original draft. **Birgit Lilletveit Kvåle:** Data curation, Formal analysis, Investigation, Methodology, Software, Validation. **Linda Andersen:** Investigation, Methodology, Validation. **Lindsey Jane Moore:** Investigation, Methodology, Validation. **Simon MacKenzie:** Conceptualization, Funding acquisition, Investigation, Methodology, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The datasets presented in this study are accessible through online repositories. The SRA submission number is SUB13917916.

Acknowledgements

This work was supported by the Norwegian Seafood Research Fund (FHF) under grant 901514. In addition, AE was funded by a full Ph.D. scholarship (Newton-Mosharafa Fund) from the Ministry of Higher Education of the Arab Republic of Egypt and the British Council.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.740455.

References

Aaen, S.M., Helgesen, K.O., Bakke, M.J., Kaur, K., Horsberg, T.E., 2015. Drug resistance in sea lice: a threat to salmonid aquaculture. Trends Parasitol. 31 (2), 72–81. https://doi.org/10.1016/j.pt.2014.12.006.

Andrews, M., Horsberg, T.E., 2021. In vitro bioassay methods to test the efficacy of thermal treatment on the salmon louse, *Lepeophtheirus salmonis*. Aquaculture 532, 736013. https://doi.org/10.1016/j.aquaculture.2020.736013.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. B. Methodol. 57 (1), 289–300.

Berland, B., Margolis, L., 1983. The early history of 'Lakselus' and some nomenclatural questions relating to copepod parasites of salmon. Sarsia 68 (4), 281–288. https://doi.org/10.1080/00364827.1983.10420582.

Bowman, J.P., 2006. The genus Psychrobacter. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E. (Eds.), The Prokaryotes: A Handbook on the Biology of Bacteria Volume 6: Proteobacteria: Gamma Subclass. Springer New York, New York, NY.

Bui, S., Madaro, A., Nilsson, J., Fjelldal, P.G., Iversen, M.H., Brinchman, M.F., Venås, B., Schrøder, M.B., Stien, L.H., 2022. Warm water treatment increased mortality risk in salmon. Veterin. Animal Sci. 17, 100265 https://doi.org/10.1016/j.vas.2022.100265.

Burridge, L., Weis, J.S., Cabello, F., Pizarro, J., Bostick, K., 2010. Chemical use in salmon aquaculture: a review of current practices and possible environmental effects. Aquaculture 306 (1), 7–23. https://doi.org/10.1016/j.aquaculture.2010.05.020.

Clokie, B.G.J., Elsheshtawy, A., Albalat, A., Nylund, A., Beveridge, A., Payne Chris, J., MacKenzie, S., 2022. Optimization of low-biomass sample collection and quantitative PCR-based titration impact 16S rRNA microbiome resolution. Microbiol. Spect. 10 (6) https://doi.org/10.1128/spectrum.02255-22 e02255–22.

Culling, C., Allison, R., Barr, W., 1985. Haematoxylin and its counterstain. In: CFA Culling (Ed.), In 'Cellular Pathology Technique', 4th edn. Butterworths-Heinemann, London, UK, pp. 111–152.

Elliott, J.M., Elliott, J.A., 1995. The effect of the rate of temperature increase on the critical thermal maximum for parr of Atlantic salmon and brown trout. J. Fish Biol. 47 (5), 917–919. https://doi.org/10.1111/j.1095-8649.1995.tb06014.x.

Elsheshtawy, A., Clokie, B.G.J., Albalat, A., Beveridge, A., Hamza, A., Ibrahim, A., MacKenzie, S., 2021. Characterization of external mucosal microbiomes of Nile Tilapia and Grey mullet co-cultured in semi-intensive pond systems. Front. Microbiol. 12 https://doi.org/10.3389/fmicb.2021.773860.

Fast, M.D., Johnson, S.C., Eddy, T.D., Pinto, D., Ross, N.W., 2007. Lepeophtheirus salmonis secretory/excretory products and their effects on Atlantic salmon immune gene regulation. Parasite Immunol. 29 (4), 179–189. https://doi.org/10.1111/j.1365-3024.2007.00932.x.

Foster, Z.S., Sharpton, T.J., Grünwald, N.J., 2017. Metacoder: an R package for visualization and manipulation of community taxonomic diversity data. PLoS Comput. Biol. 13 (2), e1005404.

Garseth, Å.H., Fritsvold, C., Svendsen, J.C., Bang Jensen, B., Mikalsen, A.B., 2018. Cardiomyopathy syndrome in Atlantic salmon Salmo salar L.: a review of the current state of knowledge. J. Fish Dis. 41 (1), 11–26. https://doi.org/10.1111/jfd.12735.

Gismervik, K., Nielsen, K., Lind, M., Vijugrein, H., 2017. Norwegian Veterinary Institute's Report Series: 6–2017. In: Mekanisk avulsing med FLS-avlusersystem= dokumentasjon av fiskevelferd og effekt mot lus. Norwegian Veterinary Institute.

Gismervik, K., Gåsnes, S.K., Gu, J., Stien, L.H., Madaro, A., Nilsson, J., 2019. Thermal injuries in Atlantic salmon in a pilot laboratory trial. Veterin. Animal Sci. 8, 100081 https://doi.org/10.1016/j.vas.2019.100081.

Grøntvedt, R.N., Nerbøvik, I.-K.G., Viljugrein, H., Lillehaug, A., Nilsen, H., Gjevre, A.-G., 2015. Thermal de-licing of salmonid fish–documentation of fish welfare and effect. Norweg. Veterin. Instit. Report Ser. 13, 2015.

Gunnarsson, G.S., Blindheim, S., Karlsbakk, E., Plarre, H., Imsland, A.K., Handeland, S., Sveier, H., Nylund, A., 2017. Desmozoon lepeophtherii (microsporidian) infections and pancreas disease (PD) outbreaks in farmed Atlantic salmon (Salmo salar L.). Aquaculture 468, 141–148. https://doi.org/10.1016/j.aquaculture.2016.09.035.

Hamre, L.A., Eichner, C., Caipang, C.M.A., Dalvin, S.T., Bron, J.E., Nilsen, F., Boxshall, G., Skern-Mauritzen, R., 2013. The Salmon louse Lepeophtheirus salmonis (Copepoda: Caligidae) life cycle has only two Chalimus stages. PLoS One 8 (9), e73539. https://doi.org/10.1371/journal.pone.0073539.

Hamre, L.A., Bui, S., Oppedal, F., Skern-Mauritzen, R., Dalvin, S., 2019. Development of the salmon louse Lepeophtheirus salmonis parasitic stages in temperatures ranging from 3 to 24°C. Aquac. Environ. Interact. 11, 429–443.

Heggland, E.I., Dondrup, M., Nilsen, F., Eichner, C., 2020. Host gill attachment causes blood-feeding by the salmon louse (Lepeophtheirus salmonis) chalimus larvae and alters parasite development and transcriptome. Parasit. Vectors 13 (1), 225. https://doi.org/10.1186/s13071-020-04096-0.

Hjeltnes, B., Bang-Jensen, B., Bornø, G., Haukaas, A., Walde, C., 2018. Fiskehelserapporten 2017 (Fish health report 2017), p. 108.

Kassambara, A., 2020a. ggplot2. Based publication ready plots [R package ggpubr version 0.2. 5].

Kassambara, A., 2020b. RSTATIX. Pipe-friendly framework for basic statistical test. https://rpkgs.datanovia.com/rstatix/.

Legrand, T.P.R.A., Catalano, S.R., Wos-Oxley, M.L., Stephens, F., Landos, M., Bansemer, M.S., Stone, D.A.J., Qin, J.G., Oxley, A.P.A., 2018. The inner workings of the outer surface: skin and gill microbiota as indicators of changing gut health in yellowtail kingfish. Front. Microbiol. 8 (2664) https://doi.org/10.3389/fmicb.2017.02664.

Lin, J., Meng, Y., Shi, Y., Lin, X., 2019. Complete genome sequences of Colwellia sp. Arc7-635, a denitrifying bacterium isolated from Arctic seawater. Curr. Microbiol. 76 (9), 1061–1065. https://doi.org/10.1007/s00284-019-01714-8.

Ljungfeldt, L.E.R., Quintela, M., Besnier, F., Nilsen, F., Glover, K.A., 2017. A pedigree-based experiment reveals variation in salinity and thermal tolerance in the salmon louse, *Lepeophtheirus salmonis*. Evol. Appl. 10 (10), 1007–1019. https://doi.org/10.1111/eva.12505.

Llewellyn, M.S., Leadbeater, S., Garcia, C., Sylvain, F.E., Custodio, M., Ang, K.P., Powell, F., Carvalho, G.R., Creer, S., Elliot, J., Derome, N., 2017. Parasitism perturbs the mucosal microbiome of Atlantic Salmon. Sci. Rep. 7, 43465. https://doi.org/10.1038/srep43465.

Longmire, J.L., Maltbie, M., Baker, R.J., 1997. Use of" Lysis Buffer" in DNA Isolation and its Implication for Museum Collections. Museum of Texas Tech University.

Lowrey, L., Woodhams, D.C., Tacchi, L., Salinas, I., 2015. Topographical mapping of the rainbow trout (Oncorhynchus mykiss) microbiome reveals a diverse bacterial community with antifungal properties in the skin. Appl. Environ. Microbiol. 81 (19), 6915–6925. https://doi.org/10.1128/AEM.01826-15.

McCarthy, E., Cunningham, E., Copley, L., Jackson, D., Johnston, D., Dalton, J.P., Mulcahy, G., 2012. Cathepsin L proteases of the parasitic copepod, Lepeophtheirus salmonis. Aquaculture 356-357, 264–271. https://doi.org/10.1016/j.aquaculture.2012.05.007.

McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8 (4), e61217. https://doi.org/10.1371/journal.pone.0061217.

Moltumyr, L., Gismervik, K., Gu, J., Gåsnes, S.K., Kristiansen, T.S., Rønnestad, I., Nilsson, J., Stien, L.H., 2021. Does the thermal component of warm water treatment inflict acute lesions on Atlantic salmon (Salmo salar)? Aquaculture 532, 736048. https://doi.org/10.1016/j.aquaculture.2020.736048.

Moltumyr, L., Nilsson, J., Madaro, A., Seternes, T., Winger, F.A., Rønnestad, I., Stien, L. H., 2022. Long-term welfare effects of repeated warm water treatments on Atlantic salmon (Salmo salar). Aquaculture 548, 737670. https://doi.org/10.1016/j.aquaculture.2021.737670.

Nilsson, J., Moltumyr, L., Madaro, A., Kristiansen, T.S., Gåsnes, S.K., Mejdell, C.M., Gismervik, K., Stien, L.H., 2019. Sudden exposure to warm water causes instant behavioural responses indicative of nociception or pain in Atlantic salmon. Veterin. Animal Sci. 8, 100076 https://doi.org/10.1016/j.vas.2019.100076.

Nilsson, J., Barrett, L.T., Mangor-Jensen, A., Nola, V., Harboe, T., Folkedal, O., 2023. Effect of water temperature and exposure duration on detachment rate of salmon lice (Lepeophtheirus salmonis); testing the relevant thermal spectrum used for delousing. Aquaculture 562, 738879. https://doi.org/10.1016/j.aquaculture.2022.738879.

Novak, C.W., Lewis, D.L., Collicutt, B., Verkaik, K., Barker, D.E., 2016. Investigations on the role of the salmon louse, Lepeophtheirus salmonis (Caligidae), as a vector in the transmission of Aeromonas salmonicida subsp. salmonicida. J. Fish Dis. 39 (10), 1165–1178. https://doi.org/10.1111/jfd.12449.

Oelckers, K., Vike, S., Duesund, H., Gonzalez, J., Wadsworth, S., Nylund, A., 2014. Caligus rogercresseyi as a potential vector for transmission of infectious Salmon Anaemia (ISA) virus in Chile. Aquaculture 420-421, 126–132. https://doi.org/10.1016/j.aquaculture.2013.10.016.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'hara, R., Simpson, G.L., Solymos, P., Stevens, M.H.H., Wagner, H., 2013. Package 'vegan'. Community Ecology Package, Version, 2(9), pp. 1–295.

Østevik, L., Stormoen, M., Evensen, Ø., Xu, C., Lie, K.-I., Nødtvedt, A., Rodger, H., Skagøy, A., Manji, F., Alarcón, M., 2022. Effects of thermal and mechanical delousing on gill health of farmed Atlantic salmon (Salmo salar L.). Aquaculture 552, 738019. https://doi.org/10.1016/j.aquaculture.2022.738019.

Overton, K., Dempster, T., Oppedal, F., Kristiansen, T.S., Gismervik, K., Stien, L.H., 2019. Salmon lice treatments and salmon mortality in Norwegian aquaculture: a review. Rev. Aquac. 11 (4), 1398–1417. https://doi.org/10.1111/raq.12299.

Pike, A.W., Wadsworth, S.L., 1999. Sealice on salmonids: Their biology and control. In: Baker, J.R., Muller, R., Rollinson, D. (Eds.), Advances in Parasitology. Academic Press.

Poppe, T., Dalum, A., Røislien, E., Nordgreen, J., Helgesen, K., 2018. Termisk behandling av laks. Norsk Veterinærtidssk. 130 (3), 148–156.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41 (D1), D590–D596. https://doi.org/10.1093/nar/gks1219.

Rebl, A., Korytář, T., Borchel, A., Bochert, R., Strzelczyk, J.E., Goldammer, T., Verleih, M., 2020. The synergistic interaction of thermal stress coupled with overstocking strongly modulates the transcriptomic activity and immune capacity of rainbow trout (Oncorhynchus mykiss). Sci. Rep. 10 (1), 14913. https://doi.org/10.1038/s41598-020-71852-8.

Roth, B., 2016. Avlusing av laksefisk med Optilice: Effekt på avlusing og fiskevelferd. Nofima Rapport 59 (2016), 41.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F., 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75 (23), 7537. https://doi.org/10.1128/AEM.01541-09.

Skiftesvik, A.B., Blom, G., Agnalt, A.-L., Durif, C.M.F., Browman, H.I., Bjelland, R.M., Harkestad, L.S., Farestveit, E., Paulsen, O.I., Fauske, M., Havelin, T., Johnsen, K., Mortensen, S., 2014. Wrasse (Labridae) as cleaner fish in salmonid aquaculture – the Hardangerfjord as a case study. Mar. Biol. Res. 10 (3), 289–300. https://doi.org/10.1080/17451000.2013.810760.

Strzyżewska, E., Szarek, J., Babinska, I., 2016. Morphologic evaluation of the gills as a tool in the diagnostics of pathological conditions in fish and pollution in the aquatic environment: a review. Vet. Med. 61, 123–132. https://doi.org/10.17221/8763-VETMED.

Sviland Walde, C., Bang Jensen, B., Pettersen, J.M., Stormoen, M., 2021. Estimating cage-level mortality distributions following different delousing treatments of Atlantic salmon (salmo Salar) in Norway. J. Fish Dis. 44 (7), 899–912. https://doi.org/10.1111/jfd.13348.

Takeuchi, M., Fujiwara-Nagata, E., Katayama, T., Suetake, H., 2021. Skin bacteria of rainbow trout antagonistic to the fish pathogen Flavobacterium psychrophilum. Sci. Rep. 11 (1), 7518. https://doi.org/10.1038/s41598-021-87167-1.

Treasurer, J., Feledi, T., 2014. The physical condition and welfare of five species of wild-caught wrasse stocked under aquaculture conditions and when stocked in Atlantic Salmon, Salmo salar, production cages. J. World Aquacult. Soc. 45 (2), 213–219. https://doi.org/10.1111/jwas.12099.

Wickham, H., 2016. Programming with ggplot2. In: Wickham, H. (Ed.), ggplot2: Elegant Graphics for Data Analysis. Springer International Publishing, Cham.