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# Experimental challenge of Atlantic salmon *Salmo salar* using clones of *Paramoeba perurans*, *P. pemaquidensis* and *Tetramitus* sp.

**Are Nylund[1,*], Martin Røed[1], Steffen Blindheim[2], Christiane Trösse[1], Linda Andersen[2]**

[1]Department of Biology University of Bergen, 5020 Bergen, Norway
[2]The Industrial and Aquatic Laboratory (ILAB), Thormøhlensgt 55, 5006 Bergen, Norway

ABSTRACT: Salmon gill disease in Norway is in most cases associated with a range of different pathogens, stress and environmental factors. *Paramoeba perurans* and other amoebae have been isolated during such disease outbreaks. Other amoebae isolated from salmon with gill disease in Norway include *P. pemaquidensis, Tetramitus* sp. and *Vannella* sp. Here we tested the pathogenicity of the first 2 species in challenge experiments. We found that even when clonal cultures of *P. pemaquidensis* established an infection on the gills of salmon, it failed to cause gill disease, while *Tetramitus* sp. appeared to be unable to establish a lasting infection on the gills of healthy salmon. The result of the challenge with *P. pemaquidensis* confirms the results of similar studies performed in the USA and in Australia. *Tetramitus* sp. is probably a common amoeba in the marine environment, and its presence on the gills of farmed salmon may just be accidental. Based on this study, we conclude that *P. perurans* is the only known amoeba in marine salmon farming associated with amoebic gill disease in Norway.

KEY WORDS: *Salmo salar* · Amoebic gill disease · *Paramoeba pemaquidensis* · *P. perurans* · *Tetramitus* sp. · *Vannella* sp. · Virulence

## 1. INTRODUCTION

Gill disease (GD) of Atlantic salmon *Salmo salar* has been an increasing problem in Norwegian aquaculture since 2000 (Nylund et al. 2007, 2008, 2011, Steinum et al. 2008). Damage to the gills may lead to respiratory and circulatory problems, resulting in reduced fish welfare and growth, and in some cases increased mortality (Powell et al. 2000). Farmers in western Norway often experience increased mortalities in the farms during stressful periods, including handling of the fish and in connection with mechanical treatments of salmon with lice infections (Overton et al. 2019, Walde et al. 2021, A. Nylund pers. obs.). The causes for GD are complex, and a range of different pathogens (viruses, bacteria and microparasites) are associated with these gill health problems. The most common pathogens associated with GD in Norway are *Paranucleospora theridion* (syn. *Desmozoon lepeophtheirii*), *Paramoeba perurans* (syn. *Neoparamoeba perurans*), *Ichthyobodo salmonis*, *Tenacibaculum maritimum*, *Candidatus* Branchiomonas cysticola, other epitheliocystis-causing agents (*C.* Piscichlamydia salmonis, *C.* Syngnamydia salmonis, *C.* Clavichlamydia salmonicola) and salmonid gill pox virus (SGPV) (Nylund et al. 2006, 2008, 2010, 2011, 2015, Karlsen et al. 2008, Isaksen et al. 2011, Mitchell et al. 2013, Småge et al. 2016, Downes et al. 2018). An addi-

*Corresponding author: are.nylund@uib.no



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tional microparasite (*Parvicapsula pseudobranchicola*), associated with pseudobranchs and gills of salmon, is causing problems in northern Norway (Karlsbakk et al. 2002, Nylund et al. 2018a).

It was initially believed that *Paramoeba pemaquidensis* caused amoebic gill disease (AGD) in salmon, and it has also been suggested that other amoebae such as *Platyamoeba* spp. could play a role in development of AGD (Kent et al. 1988, Douglas-Helders et al. 2000, Munday et al. 2001, Bermingham & Mulcahy 2007). AGD was induced under laboratory conditions by exposing naïve salmon to AGD-affected salmon or by exposing naïve salmon to purified amoeba trophozoites from AGD-affected salmon (Zilberg et al. 2001, Morrison et al. 2004). In a challenge experiment, isolates of *P. pemaquidensis* obtained from salmon with AGD failed to cause AGD in naïve salmon (Kent et al. 1988, Morrison et al. 2005). After the discovery and characterization of *Paramoeba perurans* from gills of farmed salmon in Australia and Norway, it was thought that this is the primary causative agent of AGD (Nylund et al. 2007, 2008, Young et al. 2007, 2008, Steinum et al. 2008). Later challenge experiments showed that inoculums containing clonal cultures of *P. perurans* can reproduce AGD in farmed salmon (Crosbie et al. 2012, Røed 2016, Kindt 2017, Dahle et al. 2020). *P. perurans* has been detected in marine fish aquaculture worldwide (Steinum et al. 2008, Young et al. 2008, Bustos et al. 2011, Karlsbakk et al. 2013, Mouton et al. 2014, Oldham et al. 2016, Haugland et al. 2017, Kim et al. 2017).

AGD is characterized by increased mucus production and hyperplasia causing white patches on the gills and resulting in reduced respiratory surface area (Adams & Nowak 2004, Powell et al. 2015). The hyperplasia may result in interlamellar fusion and sometimes the formation of vesicles containing *P. perurans*. The resulting gill damage/changes have, during the last decade, been classified using a gill score system (Taylor et al. 2009).

In the years following the first reports of AGD in 2006 there were no reports of GD caused by *P. perurans* in western Norway, but since 2012, AGD has been a constant challenge for salmon production in this part of the country. As part of the increased focus on GD in Norway, the Fish Diseases Research Group (FDRG) at the University of Bergen started a project on isolation of amoeba from salmon affected by GD. The major focus was on isolation and cloning of *P. perurans* from salmon with AGD, but culturing of amoebae was also attempted from salmon with GD that were negative for *P. perurans*. The result of this work led to the isolation of *Paramoeba pemaquidensis*, *Vannella* sp. and *Tetramitus* sp. from saithe *Pollachius virens*, ballan wrasse *Labrus bergylta* and Atlantic salmon. Several other amoebae had previously been identified on the gills of farmed salmon suffering from AGD in Ireland and Tasmania (Bermingham & Mulcahy 2007, English et al. 2019), but this is the first time *Tetramitus* sp. has been isolated from the gills of salmon.

The present study gives a short description of the GD associated with *P. pemaquidensis* and *Tetramitus* sp. isolated from farmed salmon and the result of challenge experiments using clones of these 2 amoebae species. Different clones of *P. perurans* were also included in the challenge experiments as positive controls.

## 2. MATERIALS AND METHODS

### 2.1. Isolation and cloning of amoebae

During a project focussing on AGD, attempts were made to isolate *Paramoeba perurans* from the gills of salmon suffering from this disease and from other GDs. Primary isolates of amoebae were obtained by adding gill tissue onto malt-yeast agar (MYA; 0.01% Bacto™ Malt Extract, 0.01% Bacto™ Yeast Extract, 2.0 Bacto™ Agar in 34‰ sterile seawater) with a layer of sterile seawater covering the tissue (Crosbie et al. 2012). The cultures were kept at 15°C, and when amoebae could be observed, the isolates were grown for 3 passages before cloning (Nylund et al. 2018b). Clonal cultures of the amoebae were established as described by Nylund et al. (2018b).

The identity of the amoebae was established by sequencing the partial small subunit (SSU) rRNA gene from cloned amoebae. Three different species of amoeba were isolated in addition to *P. perurans*. These were isolates both from salmon diagnosed with AGD and from salmon with less specific GDs in addition to cleaner fish and saithe present in salmon cages. Three isolates of *P. pemaquidensis* (clones H10/14Pq, H09/14Pq and H17/15Pq) were obtained from saithe, ballan wrasse and salmon, respectively. Ballan wrasse were used as cleaner fish for removal of salmon lice and therefore co-habitated with salmon suffering from AGD. *P. pemaquidensis* clone H09/14Pq was obtained from a saithe trapped inside a cage with salmon showing signs of GD. *Tetramitus* sp. was obtained from farmed salmon with GD in western Norway (Sogn og Fjordane and Hordaland

counties) in 2013 (clone SF05/13T) and 2015 (clone H16/15T). A *Vannella* sp. isolate was obtained from salmon lice *Lepeophtheirus salmonis* present on farmed salmon with GD in Hordaland. *Vannella* spp. have previously also been found on the gills of salmon in Australia and Ireland (Bermingham & Mulcahy 2007, English et al. 2019).

Two of these species, *P. pemaquidensis* (clones H09/14Pq and H17/15Pq) and *Tetramitus* sp. (clone H16/15T), in addition to 3 clones of *P. perurans*, were used to challenge salmon to see if they could cause AGD or GD (Table 1). The 3 *P. perurans* isolates were obtained from farmed Atlantic salmon in 2 counties (Sogn og Fjordane clone SF11/15Pp, Møre og Romsdal clone MR06/14Pp) and from ballan wrasse in Hordaland county (clone H04/14Pp). The 2 control groups received only culture media (malt-yeast broth, MYB).

Table 1. Cloned amoebae used in the challenge experiment, species, host species (isolation source) and date of isolation. For all challenges, the dosage used was 1000 amoebae $l^{-1}$, the number of salmon bath-challenged (shedders) was 30, and the number of co-habitants added 7 d after challenge was also 30

| Clone | Species | Host species | Date of isolation |
|---|---|---|---|
| H04/14Pp | *Paramoeba perurans* | *Labrus bergylta* | 27.08.2014 |
| SF11/15Pp | *P. perurans* | *Salmo salar* | 05.02.2015 |
| H09/14Pq | *P. pemaquidensis* | *Labrus bergylta* | 02.10.2014 |
| MR06/14Pp | *P. perurans* | *Salmo salar* | 18.11.2014 |
| H16/15T | *Tetramitus* sp. | *Salmo salar* | 10.09.2015 |
| H17/15Pq | *P. pemaquidensis* | *Salmo salar* | 27.08.2015 |

### 2.2. Challenge experiment

The challenge experiment was carried out at the Industrial and Aquatic Laboratory (ILAB) in Bergen in autumn 2015. The Atlantic salmon used (average weight = 127.7 g and length = 23.7 cm) came from a freshwater site in western Norway and were acclimatized to and kept at 12.0 ± 0.8°C in full seawater (salinity = 35 ± 0.1‰) in 8 tanks (150 l) with 30 specimens in each tank. An additional 240 salmon were kept in separate tanks for later use as co-habitants. Before the start of the challenge experiment, gill tissues from 30 of the salmon in the original stock were tested with respect to microparasites (viruses, bacteria and protozoans) known to be present in salmon held in freshwater at Norwegian smolt production sites. The following microparasites were included in the real-time RT-PCR test of the gill tissues: infectious salmon anaemia virus (ISAV) (Plarre et al. 2005), salmonid alphavirus (SAV) (Hodneland & Endresen 2006), *Infectious pancreatic necrosis virus* (IPNV) (Nylund et al. 2011), *Piscine orthoreovirus* (PRV1) (Nylund et al. 2018a), SGPV (Table 2), *Candidatus* Piscichlamydia salmonis (Duesund et al. 2010), *C.* Branchiomonas cysticola (Nylund et al. 2018a) and *Ichthyobodo* spp. (Isaksen et al. 2012). All 30 salmon tested were negative for the presence of these pathogens.

Table 2. Primers and probes used for real-time RT-PCR detection of *Paramoeba pemaquidensis* (Neo-uni), *Tetramitus* sp. (Tssu), *Vannella* sp. (Van) and salmonid gill pox virus (SGPV). The names of the primers and probes, their sequences, GenBank accession numbers, efficiency (=10 [−1/slope]) and product sizes (length) are shown

| Primer/probe | Sequence | Accession no. | Efficiency | Length (nt) |
|---|---|---|---|---|
| Tssu-F | AAA CGC CCG TAG TAA ACC AAA G | | | |
| Tssu-probe | ACT GAT CTG ATC GGT TCT | MT021911 | 1.98 | 70 |
| Tssu-R | CGT GGC CGA CTA TGA TGG A | | | |
| NeoUni-F | TTG TCA GAG GTG AAA TTC TTG GAT T | | | |
| NeoUni-probe | ATG AAA GAC GAA CTT CTG | MT665807 | 2.01 | 71 |
| NeoUni-R | TGA AAA CAT CTT TGG CAA ATG C | | | |
| Van-F | TCG GAA TGG TTG GCA CTT ATT | | | |
| Van-probe | GAA GAA GTA TTG GTT TGT TA | MT014020 | 1.98 | 86 |
| Van-R | CAT GCG ATC TGT TCA GTT ATT ATG AA | | | |
| SGPV-MCP-F | CAG AGG TTT TTC ATA CGC CAG AA | | | |
| SGPV-MCP-probe | TTA TAC ACC ATC ACA TTT GTG | MH061372 | 1.97 | 84 |
| SGPV-MCP-R | GAG GTC ACG GTG ATG ACA GAA C | | | |

The challenge experiments included 2 control groups (Con-I and Con-II; each group had pure/sterile culture medium instead of media containing amoebae or bacteria), 3 clones of *Paramoeba perurans*, 2 clones of *P. pemaquidensis* and 1 clone of *Tetramitus* sp. (Table 1). The salmon in each tank were challenged with 1000 amoeba $l^{-1}$ in a volume of 100 l aerated seawater. The 2 control groups were given equal amounts of culture media, equivalent to the amount of media with amoebae added to the experimental tanks. After 60 min, the water flow was reinstated, and the volume was increased to 150 l in all 8 tanks. The salmon were kept in a flow-through system during the rest of the experiment. Co-habitants (N = 30) were added 7 d post challenge (dpc) to each of the 8 tanks (Table 1). The shedders (i.e. bath-challenged salmon) in each tank were sampled at 6, 13 and 20 dpc, while the co-habitants were collected on Days 11, 18 and 25 after being added to the tanks (Fig. 1).

Weight, length and gill score were registered for all fish sampled. The gill score was calculated for both sides on all 8 gill arches (Taylor et al. 2009). A 100% score was given when all sides had a gill score = 5 (an average gill score of 3 on all 16 sides = 60%). The gill score obtained in the challenge experiment is given as a percentage of maximum score possible.

Gill tissues for real-time RT-PCR detection of the amoebae and sequencing were collected from the second gill arch of the left side, while the third gill arch on the left side was sampled for histology.

Amoebae used in the challenge experiment were re-isolated from co-habitants collected 25 dpc, as described by Morrison et al. (2004). Two gill arches with visible 'patches', indicating AGD, were sampled from the salmon. One of the gill arches was added to a cell culture flask (25 $cm^2$) containing MYB (gill arch was removed after 15 h). The second gill arch was streaked onto a petri dish (MYA) to which sterile seawater was added. The samples were checked during the following 7 d for growth of amoebae. If positive, they were tested by real-time RT-PCR and sequenced by Sanger sequencing of the SSU rRNA gene from the amoebae.

The experiment was approved by the Norwegian Food Safety Authority according to the European Union Directive 2010/63/EU for animal experiments (permit numbers 7904 and 9066). All fish were treated according to the Norwegian Animal Welfare Act (01/01/2010).

### 2.3. Histology

Gill tissues from salmon suffering from AGD or GD at marine production sites and gills from salmon challenged with *Paramoeba perurans*, *P. pemaquidensis* and *Tetramitus* sp. were fixed in a modified Karnovsky fixative (Steigen et al. 2013). The tissues were embedded in EMBED 812, and semi- and ultrathin sections were cut from the resin blocks and used for light and transmission electron microscopic (TEM) studies.

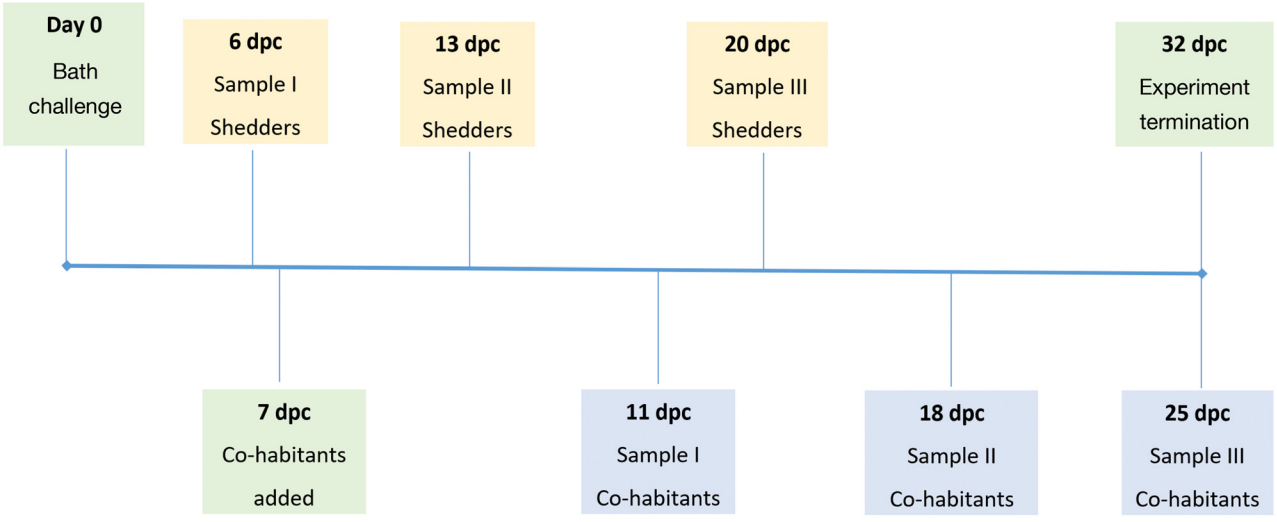


Fig. 1. Timeline for the challenge experiment. Salmon (shedders) were bath-challenged on Day 0, and co-habitants were added 7 d post challenge (dpc). Shedders were sampled (N = 10 at each sampling point) at 6, 13 and 20 dpc, while the co-habitants were sampled (N = 10 at each sampling point) at 11, 18 and 25 dpc (after being added to the tanks)

### 2.4. RNA/DNA extractions and sequencing

RNA was extracted as described by Gunnarsson et al. (2017a), and DNA was extracted using the DNeasy DNA Tissue kit (Qiagen) as recommended by the manufacturer. Elution of the DNA was performed twice in 50 µl of 10 mM Tris-HCl (pH 8.5), to increase the overall DNA yield. RNA and DNA were stored at −20°C until use.

The DNA extracted from the clonal cultures of the amoebae was used to sequence the SSU rRNA gene for identification of genus and species. In addition to 3 clonal cultures of *P. perurans,* 3 cultures of *P. pemaquidensis* (GenBank accession nos. MT665807, MT665808, MT665809), 2 cultures of *Tetramitus* sp. (MT021911) and 1 culture of *Vannella* sp. (MT014020) were identified. The *Vannella* sp. showed 99.4% sequence identity (1886 nucleotides of the rRNA SSU gene) to *V. contorta* (DQ229953) isolated from water samples obtained at 10 m depth from the north edge of the Ross Sea pack ice (Moran et al. 2007).

### 2.5. Real-time RT-PCR and normalization of data

New real-time RT-PCR assays were developed targeting the SSU rRNA of *Tetramitus* sp. and *Vannella* sp. (Table 2), while an assay targeting the SSU rRNA from *P. perurans* has already been published (Nylund et al. 2018b). *P. pemaquidensis* were detected on the gills of challenged salmon using an assay (NeoUni) targeting the SSU rRNA gene of *Paramoeba* spp. (Table 2). Standard curves for the assays were generated by using a 10-fold dilution series of RNA in triplicates, and the PCR efficiency ($E$ = 10[−1/slope]) was calculated (Nylund et al. 2018b). A previously developed assay (EF1A, E = 1.94, Olsvik et al. 2005) targeting the elongation factor 1 alpha, was used as an internal standard when running real-time RT-PCR on gill tissues.

The normalized expression (NE) of the amoebae SSU rRNA gene was calculated using the formula NE = $(E_{ref})^{Ct\ ref}$/ $(E_{target})^{Ct\ target}$. Mean NE (MNE) was calculated from the NE values.

### 2.6. Phylogeny

Preliminary species identification of the amoebae clones, using 18S rRNA (*Tetramitus* sp.: 2085 nucleotides [nt], *Vannella* sp.: 1886 nt, *P. pemaquidensis*: 1860 nt), was performed by searching the public GenBank database for related homologous sequences using BLAST (2.0). The Vector NTI Suite software package was used to generate multiple alignments of the sequences. Selected sequences from existing genera, available on the EMBL nucleotide database, were included in pairwise sequence comparisons. The multiple sequence alignment editor GeneDoc (www.psc.edu/biomed/genedoc) was used for manual adjustment of the sequence alignments (gaps in the alignments were removed). A maximum likelihood tree was generated and bootstrapped (50 000 puzzling steps) in TREE_PUZZLE 5.2 (current version; www.tree-puzzle.de). Phylogenetic trees were drawn using FigTree (v1.4.3, A. Rambaut)

## 3. RESULTS

### 3.1. Salmon gills from field samples

*Tetramitus* sp. was associated with GD in Atlantic salmon in 2 counties in western Norway: Hordaland (isolate: H16/15T) and Sogn og Fjordane (isolate: SF05/13T). Gills from the salmon in Hordaland showed areas of necrotic tissue, loss of tissue and hyperplasia (Fig. 2). Several pathogens were present in the gills of the salmon: *Paramoeba perurans* (Syn. *Neoparamoeba perurans*), *Tetramitus* sp., *Ichthyobodo salmonis*, *Paranucleospora theridion* (syn. *Desmozoon lepeophtheirii*), *Candidatus* Branchiomonas cysticola and SGPV. A few salmon were also positive for SAV. The salmon with GD in Sogn og Fjordane had pale gills, increased mucus production and gill bleeding (aneurism), and were positive for *Tetramitus* sp., *Paranucleospora theridion*, *Ichthyobodo salmonis*, *Cand*. Branchiomonas cysticola, *Tenacibaculum* spp. and SGPV (Fig. 2). Histological changes of the gill tissue and presence of putative amoebae associated with the gills are presented in Fig. 2E–H. A clonal isolate of *Tetramitus* sp. (SF05/13T) is shown in Fig. 3. All salmon from the 2 farms were also positive for PRV1. Based on phylogenetic analysis of the SSU rRNA gene (accession no. MT021911), the closest relative to the 2 *Tetramitus* sp. clones is *T. vestfoldii* isolated from Pendent Lake, Vestfold Hills, eastern Antarctica (Murtagh et al. 2002) (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d145p001_supp.pdf).

*Paramoeba pemaquidensis* were isolated from 3 different fish species (salmon, saithe and ballan wrasse). The 2 non-salmonid species were collected from net-pens containing farmed salmon. The sal-

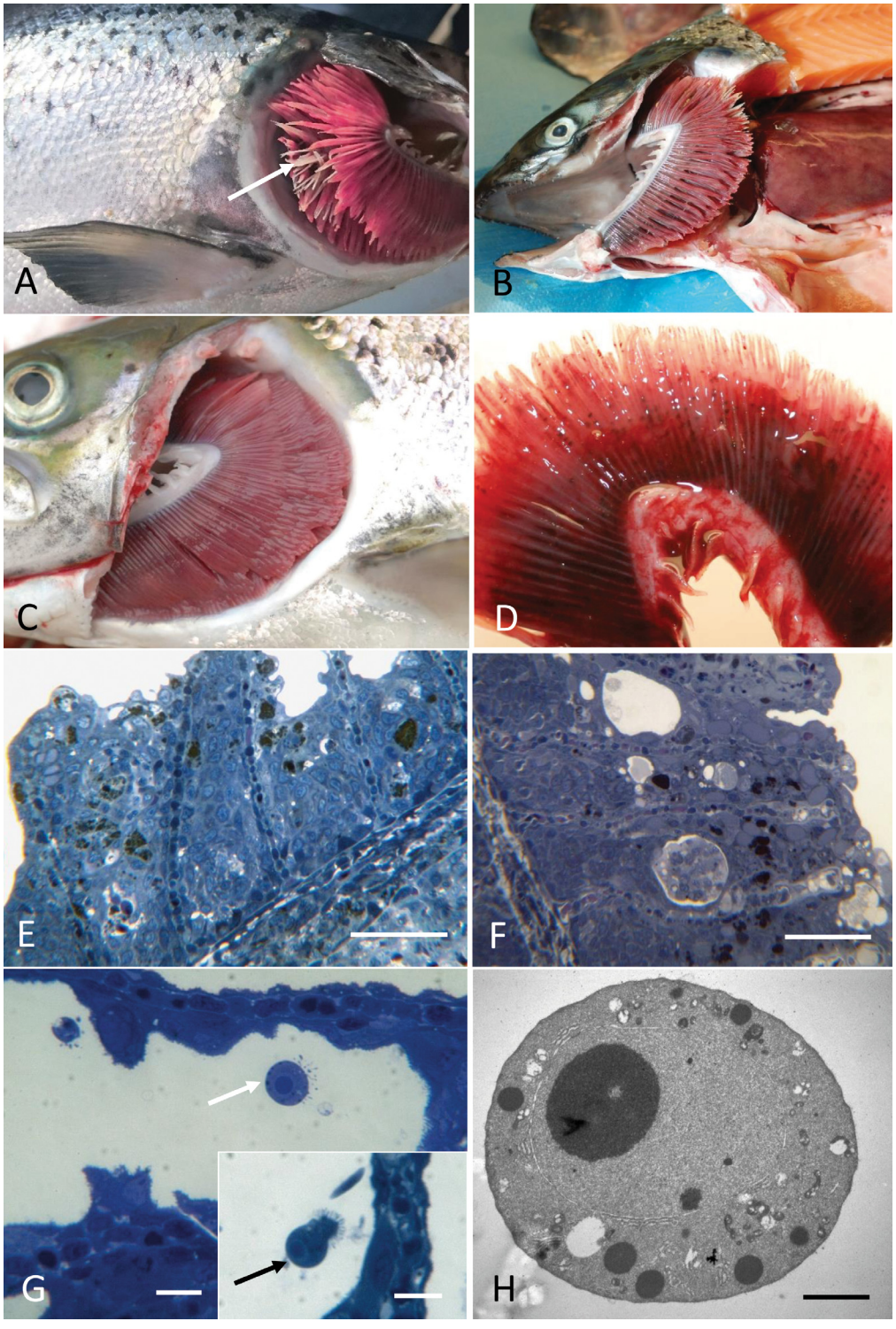


Fig. 2. Gill pathology of salmon positive for *Tetramitus* sp. (A,B) Gill pathology observed in the salmon from Hordaland county. In panel A, note the necrotic area with loss of tissue (arrow). (C,D) Gill pathology observed in the salmon from Sogn og Fjordane where some had pale gills with increased mucus production (C) while others had distinct gill bleeding (aneurisms, D). (E,F) Histopathological changes in the gills believed to be associated with *Paranucleospora theridion* (E) and *Candidatus* Branchiomonas cysticola (F) (bars = 50 µm). (G,H) Amoeba-like cells (arrows) associated with the gills of salmon suffering from gill disease (bars = 10 µm). Panel H shows a transmission electron microscopic image of these cells (note the large nucleus and the distinct nucleolus) (bar = 2.0 µm)

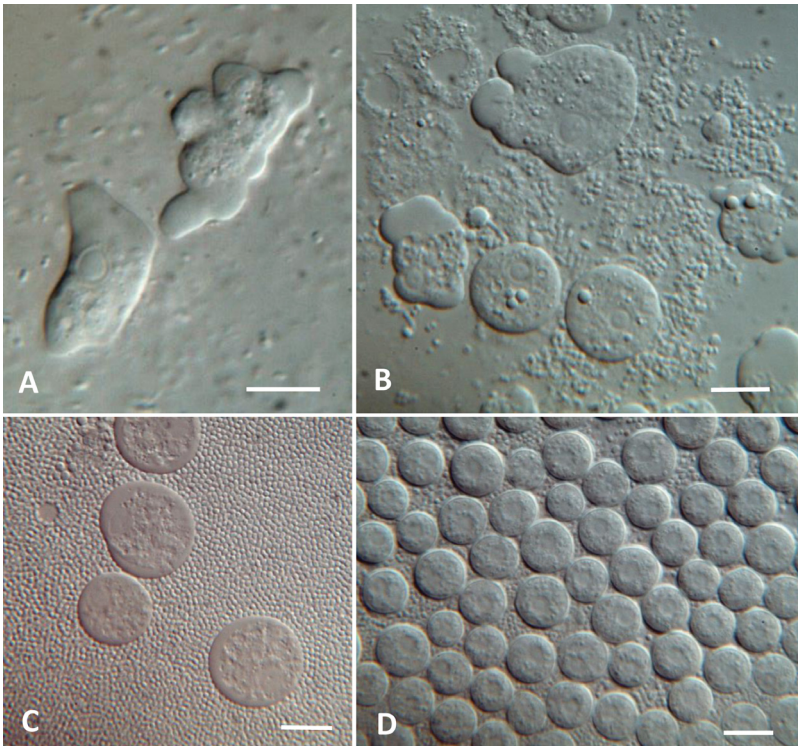


Fig. 3. Clonal culture of *Tetramitus* sp. (SF05/13T) showing (A) trophozoites, (B,C) early stages of spore formation and (D) spores (bars = 10 µm)

mon in these 3 farms were suffering from GD, and *P. perurans* were present on some of the salmon in the cages. The *P. pemaquidensis* clone H17/15Pq was isolated from salmon whose gills had patches of mucus, hyperplasia and aneurisms, and were positive for a range of pathogens, including ISAV (HPR0), IPNV, SGPV, *Cand.* Branchiomonas cysticola, *Paranucleospora theridion, Ichthyobodo salmonis, P. perurans* and *P. pemaquidensis*. The gills from saithe and ballan wrasse, from which H09/14Pq and H10/14Pq were isolated, showed no sign of GD. The morphology of the clonal isolate of *P. pemaquidensis* obtained from ballan wrasse (H09/14Pq), saithe (H10/14Pq) and salmon (H17/15Pq) is shown in Fig. 4. Phylogenetic analyses of the 3 *P. pemaquidensis* clones (H09/14Pq [MT665807], H10/14Pq [MT665808], H17/15Pq [MT665809]) show that they group close to each other within a clade containing isolates from Australia and the USA (Fig. S2).

*Vannella* sp. was isolated from salmon lice *Lepeophtheirus salmonis* that were present on salmon suffering from AGD. Using the real-time RT-PCR assay targeting *Vannella* sp. (Table 2) showed that this species was not present on the salmon gills that were heavily infected with *P. perurans*. The SSU rRNA gene from clone H13/13V (Fig. 5) of *Vannella* sp. showed a close relationship to *V. contorta* (accession no: DQ229953) isolated from seawater from the north edge of the Ross Sea pack ice (Moran et al. 2007) (Fig. S3). This clone was not used in the challenge experiments.

### 3.2. Challenge experiment

None of the salmon (shedders or co-habitants) died during the experimental period after challenge. However, both the shedders and co-habitants challenged with the 3 clones of *P. perurans* (H04/14Pp, MR06/14Pp, SF11/15Pp) developed AGD. The percentages of maximum gill score for the shedders and co-habitants sampled during the experimental period differed among the 3 clones of *P. perurans* (Fig. 6). The gill score increased from the first to the last sampling, both among the shedders and the co-habitants. In the last sampling of the shedders challenged with H04/14Pp, MR06/14Pp and SF11/15Pp, 14, 63 and 2 gill sides, respectively, reached a gill score ≥3 (out of a total of 160 sides, i.e. 16 sides in 10 individuals). Of these 3 clones, collected in 3 different counties in Norway, MR06/14Pp showed the highest virulence (gill scores) at 12°C (Fig. 6). The experiment was terminated before the gill score reached a level associated with mortality. In the 2 control groups, only 2 (Con-I) and 3 (Con-II) individuals (N = 60 for each control group) had a gill score = 1 on one side of a gill arch, respectively. The controls were negative for the presence of *P. perurans*.

Gill tissues from 10 salmon were sampled at 6, 13 and 20 dpc from the shedder groups, while the same number of salmon were sampled at 11, 18 and 25 dpc from the co-habitant groups. The results of the real-time RT-PCR screening for *P. perurans* present on the salmon gills are given in Table 3. Based on the MNE of *P. perurans* (SSU rRNA) on the gills of the salmon, in the successive samplings of the shedders, there was an increase in the number of amoebae with increasing number of dpc (Fig. 7). The MNE of *P. perurans* RNA levels in the co-habitants increased from 11 to 18 dpc, followed by a drop in the period from 18 to 25 dpc. Histopathological changes in the gills of salmon challenged with *P. perurans* included

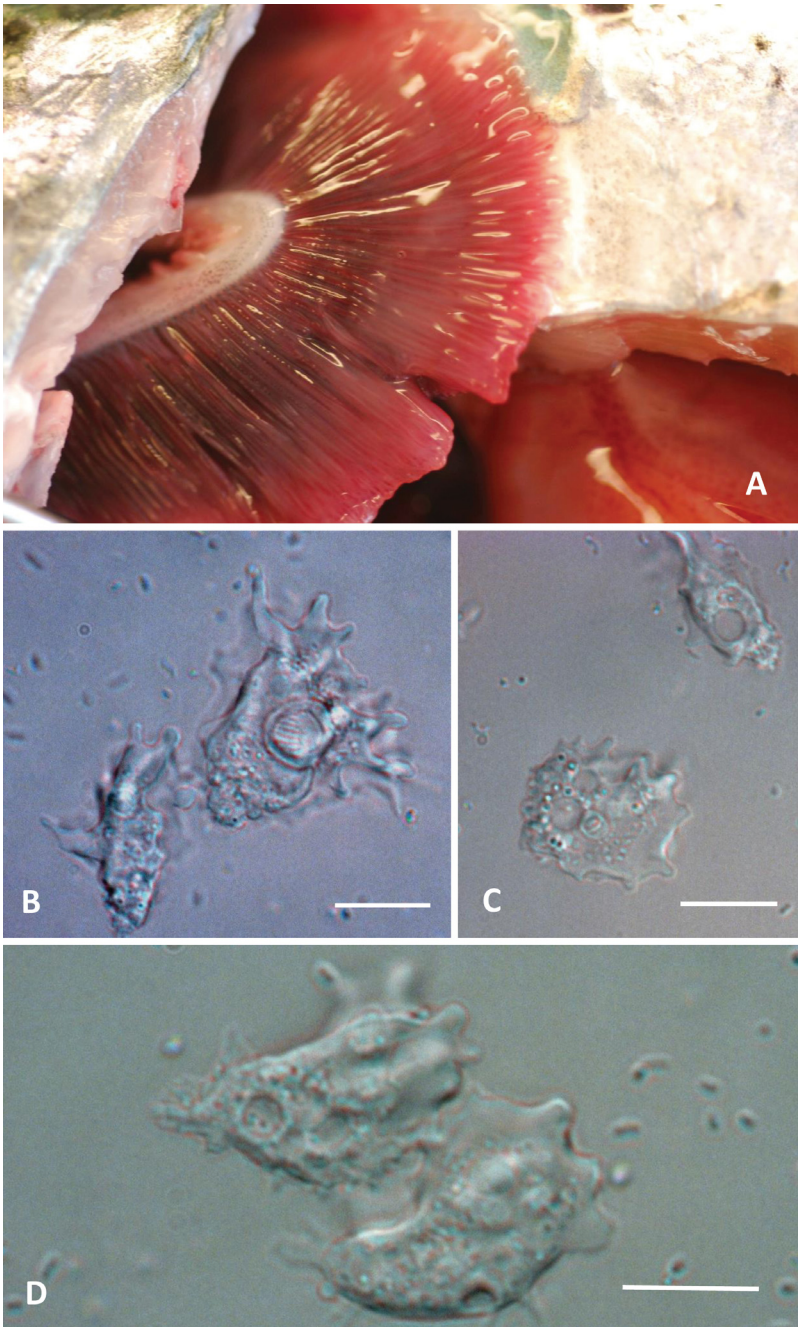


Fig. 4. (A) Gill arch from salmon diagnosed with amoebic gill disease (AGD) and positive for *Paramoeba pemaquidensis* and *P. perurans*. The gills are slightly pale and show areas with high mucus production. Also shown are trophozoites of *P. pemaquidensis* from clonal cultures obtained from (B) ballan wrasse (H09/14Pq), (C) saithe (H10/14Pq) and (D) salmon (H17/15Pq), respectively (bars = 10 µm)

increased numbers of mucus cells and hyperplasia. *P. perurans* was isolated from the salmon in all tanks challenged with clones MR06/1Pp, SF11/15Pp and H04/14Pp.

None of the groups challenged with *P. pemaquidensis* (H17/15Pq and H09/14Pq) and *Tetramitus* sp. (H16/15T) reached a total gill score above 2.0% of the maximum gill score. The highest gill score (=2) was seen on 3 gill sides of 1 salmon in the shedder group, challenged with H16/15T and collected 20 dpc. The percentage of fish with gill scores was only slightly higher in the groups challenged with *P. pemaquidensis* and *Tetramitus* sp. compared to that observed in the control groups (Fig. 8).

The result of the real-time RT-PCR testing of gills for presence of *P. pemaquidensis*, from shedders and co-habitants, using the NeoUni assay is presented in Table 3. The prevalence of *P. pemaquidensis* on the gills of shedders and co-habitants challenged with H09/14Pq was higher compared to that of the salmon challenged with H17/15Pq. The highest prevalence for both groups was observed among the co-habitants at 18 dpc, but cycle threshold ($C^T$) values (>19.0) indicate a relatively low amount of amoebae on the gills of salmon in both groups at all sampling points. Real-time RT-PCR testing of the salmon challenged with H16/15T (Tssu assay) resulted in 1 positive shedder (out of 30 total) and 1 positive co-habitant (out of 30). None of the controls were positive for any of the amoebae. Histological examination of the salmon challenged with *P. pemaquidensis* and *Tetramitus* sp. showed no changes in the gill tissues. It was not possible to isolate *P. pemaquidensis* and *Tetramitus* sp. from the challenged groups at the end of the experimental period.

## 4. DISCUSSION

*Paramoeba perurans* has caused AGD among farmed salmon in western Norway since 2012, with up to 90% mortality in some farms (A. Nylund pers. obs.). However, other gill pathogens and other pathogens are also always present in the salmon at the sites where AGD occurs.

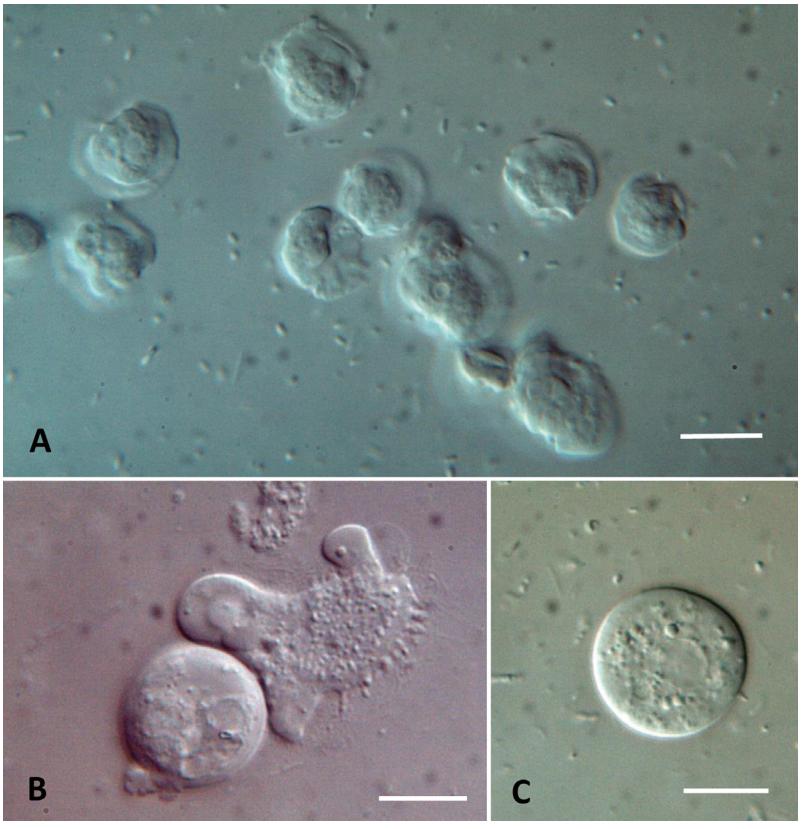


Fig. 5. (A,B) Clonal cultures (clone H13/13V) of *Vannella* sp. isolated from *Lepeophtheirus salmonis*, present on salmon with gill disease, showing motile trophonts. (C) Cyst of *Vannella* sp. (bars = 10 µm)

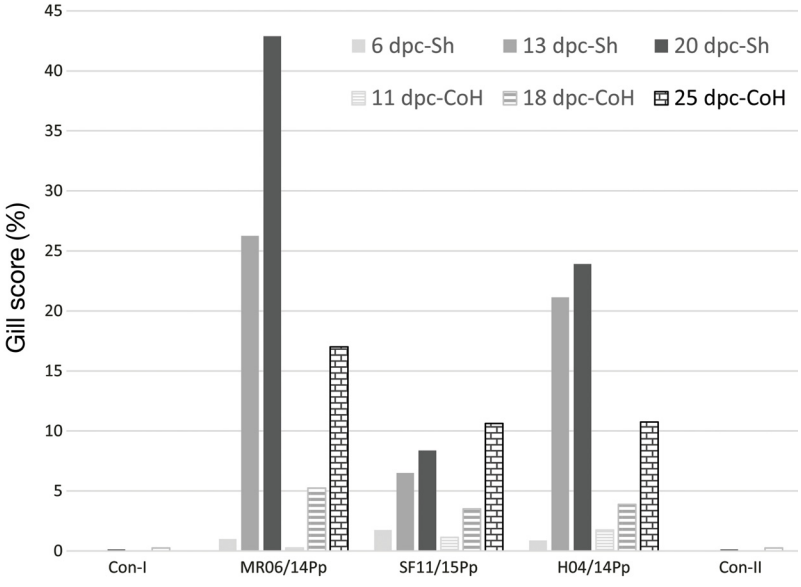


Fig. 6. Percentage gill score (*y*-axis) for 3 clones of *Paramoeba perurans* compared to the 2 control groups (Con). Sh: shedders; CoH: co-habitants; dpc: days post challenge. The gill score is presented as a percentage of the maximum gill score. N = 10 for each sampling

The dominating gill pathogens in western Norway are SGPV, low virulent ISAV (HPR0), *Paranucleospora theridion* (syn. *Desmozoon lepeophtheirii*), *Paramoeba perurans* (syn. *Neoparamoeba perurans*), *Ichthyobodo salmonis* and *Candidatus* Branchiomonas cysticola. These are common pathogens associated with GD in salmon aquaculture (Nylund et al. 2008, 2011, Downes et al. 2018, Herrero et al. 2018). In the present study, we also isolated other amoebae from farmed salmon with GD in western Norway: *Paramoeba pemaquidensis*, *Tetramitus* sp. and *Vannella* sp. *P. pemaquidensis* was isolated from ballan wrasse (isolate H09/14Pq), saithe (isolate H10/14Pq) and salmon (isolate H17/15Pq), while *Tetramitus* sp. was isolated from salmon only (isolates SF05/13T and H16/15T).

*Vannella* sp. was isolated from salmon lice, and it has been suggested that this parasite could be a vector for spreading of *P. perurans* (Nowak et al. 2010). A high diversity of *Vannella* spp. has previously been found on the gills of salmon and, even though the role that these amoebae may play in disease development is unknown, they are considered to be non-pathogenic (English et al. 2019). In our study, *Vannella* sp. was not found on any of the farmed salmon gills tested, so it was decided not to include this species in the challenge experiment.

A high diversity of amoebae associated with gills of farmed salmon have previously been observed in both Ireland and Australia (Bermingham & Mulcahy 2007, English et al. 2019), and challenge experiments using isolates of *P. pemaquidensis* have been performed (Kent et al. 1988, Morrison et al. 2005). However, our study is the first challenge experiment using clonal cultures of *P. pemaquidensis* and *Tetramitus* sp. isolated from salmon in Norway. Both *P. pemaquidensis* and *Tetrami-*

Table 3. Real-time RT-PCR detection of *Paramoeba perurans* (Pp), *P. pemaquidensis* (Pq) and *Tetramitus* sp. (T) on salmon shedders and co-habitants in the challenge experiment. The co-habitants were challenged 7 d after the shedders. Pos N = number of positive salmon/number of tested salmon; dpc: days post challenge

| Shedders | Pos N | 6 dpc | | Pos N | 13 dpc | | Pos N | 20 dpc | |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean Ct | Range of Ct | | Mean Ct | Range of Ct | | Mean Ct | Range of Ct |
| MR06/14Pp | 10/10 | 21.8 | 17.5–27.1 | 10/10 | 18.4 | 14.4–24.5 | 10/10 | 16.6 | 12.2–19.7 |
| SF11/15Pp | 10/10 | 27.8 | 24.1–33.2 | 10/10 | 21.2 | 13.9–25.4 | 10/10 | 18.2 | 11.2–23.1 |
| H04/14Pp | 10/10 | 22.1 | 19.2–26.1 | 10/10 | 16.5 | 12.9–20.1 | 10/10 | 15.2 | 10.2–19.8 |
| H16/15T | 1/10 | 36.7 | – | 0/10 | – | – | 0/10 | – | – |
| H17/15Pq | 4/10 | 28.25 | 27.8–28.9 | 4/10 | 29.7 | 28.5–31.2 | 1/10 | 29.0 | – |
| H09/14Pq | 1/10 | 24.1 | – | 8/10 | 27.2 | 23.7–28.9 | 5/9 | 27.1 | 21.6–29.5 |
| **Co-habitants** | **Pos N** | **11 dpc** | | **Pos N** | **18 dpc** | | **Pos N** | **25 dpc** | |
| | | Mean Ct | Range of Ct | | Mean Ct | Range of Ct | | Mean Ct | Range of Ct |
| MR06/14Pp | 10/10 | 22.0 | 19.0–27.6 | 10/10 | 20.7 | 15.0–26.3 | 10/10 | 18.9 | 14.8–22.2 |
| SF11/15Pp | 10/10 | 29.3 | 25.0–35.2 | 10/10 | 22.2 | 14.8–30.9 | 10/10 | 19.7 | 13.3–26.1 |
| H04/14Pp | 9/10 | 28.3 | 19.2–32.6 | 10/10 | 20.3 | 13.0–23.6 | 10/10 | 20.7 | 15.0–28.1 |
| H16/15T | 1/10 | 25–5 | – | 0/10 | – | – | 0/10 | – | – |
| H17/15Pq | 2/10 | 27.6 | 25.2–29.9 | 8/10 | 25.8 | 19.1–29.9 | 3/10 | 27.1 | 24.8–28.7 |
| H09/14Pq | 2/10 | 27.5 | 25.3–29.7 | 10/10 | 26.6 | 22.1–29.4 | 9/10 | 26.8 | 23.3–28.5 |

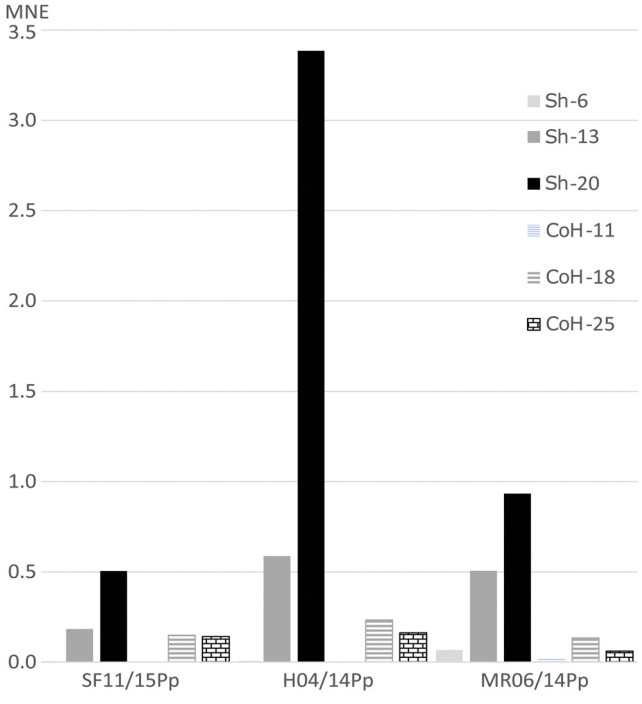


Fig. 7. Mean normalized expression (MNE) of *Paramoeba perurans* rRNA on the gills of 10 salmon collected at each time point. Sh: shedders (collected 6, 13 and 20 dpc); CoH: co-habitants (collected 11, 18 and 25 dpc)

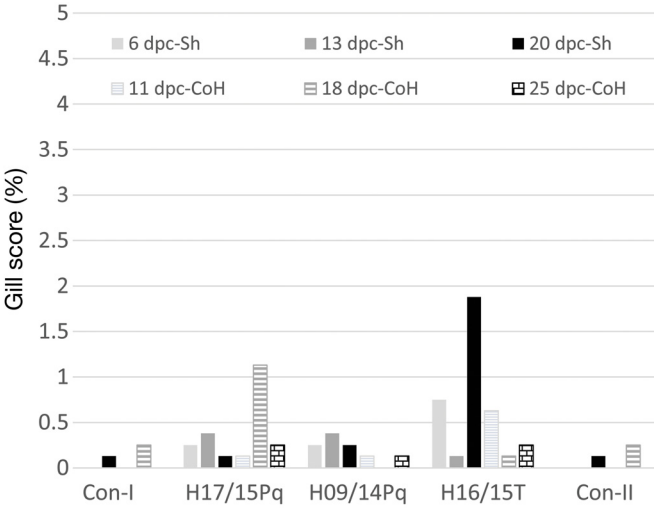


Fig. 8. Percentage gill score (*y*-axis) for 2 clones of *Paramoeba pemaquidensis* (H09/14Pq, H17/15Pq) and 1 clone of *Tetramitus* sp. (H16/15T) compared to the 2 control groups (Con). Sh: shedders; CoH: co-habitants; dpc: days post challenge. The gill score is presented as a percentage of the maximum gill score. Note that the *y*-axis only covers the range 0–5%

*tus* sp. used in the challenge were isolated from salmon with severe GD. However, they were not able to cause any GD in this experiment carried out at 12°C using pathogen-free salmon. The gill score of the salmon in the tanks during the experimental period was only slightly different from that found in the control groups, and only low amounts of *P. pemaquidensis* could be detected on the gills of challenged salmon. This finding supports previous studies suggesting that *P. pemaquidensis* in not the causative agent of AGD (Morrison et al. 2005, Young et al. 2007, 2008). To our knowledge, *Tetramitus* sp. has only been isolated from the gills of salmon in

Norway, and according to the challenge experiment, this amoeba is not able to cause AGD in a pathogen-free environment. *Tetramitus* sp. is probably a free-living non-pathogenic amoeba that can be accidently found on salmon in farms.

In the challenge experiment, clonal cultures of 3 different strains of *P. perurans* gave a distinct increase in gill score in both the shedders and the cohabitants during the experimental period. The experiment was terminated before any mortality occurred, but based on the increasing gill score during the experimental period, it is safe to conclude that *P. perurans* may cause severe AGD and mortality at 12°C even in the absence of other pathogens. It has been suggested that temperatures above 12°C represent an important risk factor for AGD outbreaks (Benedicenti et al. 2019), and in Norway, the majority of AGD outbreaks occur in the autumn at temperatures between 15 and 18°C. However, the present study shows that *P. perurans* may also cause AGD at 12°C with a gill score as high as 4 on individual gill arches 20 dpc. The increase in gill score coincided with an increase in the amount of *P. perurans* rRNA detected by real-time RT-PCR at the same sampling points. The *P. perurans* clone causing the highest gill score (MR06/14Pp) was isolated from salmon in the northern part of western Norway. These results suggest that *P. perurans* has the potential to spread north of western Norway and may even cause AGD in the southern parts of northern Norway, where temperatures are higher than 12°C during summer and autumn. AGD caused by *P. perurans* has been observed in February at 9°C in a semi-closed containment system in western Norway (A. Nylund pers. obs.).

## 5. CONCLUSIONS

The present study gives no support to the hypothesis that *Paramoeba pemaquidensis* and *Tetramitus* sp. are important factors for the development of GD among Norwegian farmed salmon. *P. perurans* seems to be the only amoeba isolated from gills of farmed salmon and marine fish in Norway that is able to cause AGD, confirming several other studies performed during the last decade (Crosbie et al. 2012, Bridle et al. 2015, Røed 2016, Chalmers et al. 2017, Kindt 2017, Dahle et al. 2020).

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