Repeated sub-lethal freshwater exposures reduce the amoebic gill disease parasite, *Neoparamoeba perurans*, on Atlantic salmon

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Abstract

Freshwater bathing is one of the main treatment options available against amoebic gill disease (AGD) affecting multiple fish hosts in mariculture systems. Prevailing freshwater treatments are designed to be long enough to kill *Neoparamoeba perurans*, the ectoparasite causing AGD, which may select for freshwater tolerance. Here, we tested whether using shorter, sub-lethal freshwater treatment durations are a viable alternative to lethal ones for *N. perurans* (2−4 h). Under *in vitro* conditions, gill-isolated *N. perurans*...
attached to plastic substrate in seawater lifted off after ≥ 2 min in freshwater, but survival was not impacted until 60 min. In an in vivo experiment, AGD-affected Atlantic salmon _Salmo salar_ subjected daily to 30 min (sub-lethal to _N. perurans_) and 120 min (lethal to _N. perurans_) freshwater treatments for 6 days consistently reduced _N. perurans_ cell numbers on gills (based on qPCR analysis) compared to daily 3 min freshwater or seawater treatments for 6 days. Our results suggest that targeting cell detachment rather than cell death with repeated freshwater treatments of shorter duration than typical baths could be used in AGD management. However, the consequences of modifying the intensity of freshwater treatment regimes on freshwater tolerance evolution in _N. perurans_ populations requires careful consideration.

**KEYWORDS** _Neoparamoeba perurans_, freshwater, moderate vs aggressive treatment, parasite control, mariculture

**1 INTRODUCTION**

Amoebic gill disease (AGD) is an emerging concern for global finfish mariculture (Oldham, Rodger & Nowak, 2016). AGD outbreaks are most effectively controlled using freshwater bathing treatments. This is suitable for 8 of the 16 AGD-affected fish species used in mariculture which are freshwater-tolerant (ayu _Plecoglossus altivelis_, sea bass _Dicentrarchus labrax_, flathead grey mullet _Mugil cephalus_ and 5 salmonid species) (Froese & Pauly, 2018; Oldham et al., 2016; Kim, Kong, Kim, Jung & Oh, 2017). However, freshwater bathing is expensive and difficult to apply in areas where freshwater access is limited (Munday, Zilberg & Findlay, 2001; Rodger, 2014). As a consequence, hydrogen peroxide in seawater baths is sometimes used despite risking mortalities, particularly at high temperatures (Overton, Samsing, Oppedal, Dalvin, Stien & Dempster, 2018; Overton, Samsing, Oppedal, Stien & Dempster, 2017) and when AGD levels are severe in cage populations (Adams, Crosbie & Nowak, 2012; Rodger, 2014). Both bathing practices can also negatively affect salmon growth (Douglas-Helders, Weir, O’Brien, Carson & Nowak, 2004) and welfare (Adams et al., 2012; Overton et al., 2018; Overton et al., 2017).
Another fundamental problem with current freshwater treatments that seek to kill the *Neoparamoeba perurans*, the ectoparasite responsible for AGD, is that they are partially effective; leaving some surviving amoebae on gills (Adams et al., 2012; Parsons, Nowak, Fisk & Powell, 2001). This contributes to rapid re-infections that require subsequent treatment (sometimes as soon as 1-week post-bath) (Clark, Powell & Nowak, 2003). Lima et al. (2017) also noted that small proportions of gill-isolated *N. perurans* populations on plastic substrate withstand freshwater treatments at durations thought to be lethal and widely used in commercial salmon farming. Presumably, the more freshwater tolerant *N. perurans* are strongly selected for and contribute traits to subsequent generations, resulting in evolution of freshwater tolerance. For sea-caged Atlantic salmon *Salmo salar*, this may underlie increased freshwater bathing frequency against AGD, rising from 3–4 baths (Clark & Nowak, 1999) to as many as 15 baths per production cycle in SE Tasmania over ~ 15 years (Rodger, 2014). However, increasing bath frequency may be linked to more intense AGD outbreaks over time due to higher stocking densities of Atlantic salmon farming areas in SE Tasmania, or climate change effects (Leung & Bates, 2013).

Recent evidence suggests that shorter, sub-lethal freshwater exposures may also affect *N. perurans* populations. In a preliminary investigation, Lima et al. (2017) observed that under *in vitro* conditions, gill-isolated *N. perurans* detached from plastic substrate within approximately 5 min and formed a floating pseudocyst to cope with short-term reductions in salinity, as in other marine amoebae (Cowie & Hannah, 2006). This occurred well before 2 h which is when *N. perurans* survival is affected with freshwater exposures under these conditions (Lima et al., 2017; Powell & Clark, 2003). The interplay between the removal of *N. perurans* as they form floating pseudocysts in freshwater (Lima et al., 2017), along with the shedding of mucus and cellular debris (Adams & Nowak, 2004; Clark et al., 2003; Parsons et al., 2001), reveal the possibility of using sub-lethal freshwater exposures in AGD management. However, shorter, and in effect less aggressive, freshwater treatments may change the rates of freshwater tolerance evolution in *N. perurans* populations (Colijn & Cohen, 2015; Hughes & Andersson, 2017; Kouyos, Metcalf, Birger, Klein, Abel zur Wiesch, Ankomah, Arinaminpathy, Bogich, Bonhoeffer, Brower, Chi-Johnston, Cohen, Day, Greenhouse, Huijben, Metlay, Mideo, Pollitt, Read, Smith, Standley, Wale & Grenfell, 2014).

Novel approaches to parasite treatment that use fish behaviour and cage environment manipulations to minimise handling and fish welfare impacts are gaining traction in
Atlantic salmon sea-cage farming for other parasitic diseases (Bui, Oppedal, Sievers & Dempster, 2017). For example, freshwater or low salinity surface environments have been created over long timeframes in sea-cages, which may reduce freshwater-sensitive parasites, depending on the salinity changes that salmon experience (Wright, Stien, Dempster, Vågseth, Nola, Fosseidengen & Oppedal, 2017b). Physostomous (open swim-bladder) fish such as salmonids (Fahlén, 1971) frequently refill their swim bladder by surfacing for air through such a surface environment (Dempster, Kristiansen, Korsøen, Fosseidengen & Oppedal, 2011). Atlantic salmon may also move into surface layers for extended periods at night and during feeding or resulting from other motivational factors (Oppedal, Dempster & Stien, 2011). In commercial snorkel cages, continuous filling with freshwater to create surface salinities down to 4–5 g L\(^{-1}\) has coincided with reduced AGD levels (Wright et al., 2017b). Developing these methods for AGD management requires a more detailed understanding of how freshwater affects \(N. perurans\) populations.

Here, in an \textit{in vitro} experiment, we tested how freshwater exposure duration at a fine temporal scale altered the detachment and survival of gill-isolated \(N. perurans\) attached to plastic petri dishes. Further, in an \textit{in vivo} experiment, we determined if daily repetition of different freshwater exposure durations altered \(N. perurans\) populations on the gills of AGD-affected Atlantic salmon. The range of exposure frequencies and durations were selected to cover possible exposures salmon may spend in a continuously deployed freshwater or low salinity surface layer created in sea-cages within 24 h periods.

### 2 MATERIALS AND METHODS

#### 2.1 Experiment 1 – Effect of freshwater exposure duration on gill-isolated \(N. perurans\)

Both experiments were performed at the Institute of Marine and Antarctic Studies (IMAS), University of Tasmania. \(N. perurans\) amoebae were harvested from the gills of AGD-affected salmon from an AGD infection tank as described in Morrison et al. (2004), centrifuged (500 \(g\), 5 min, \(4\)\(^\circ\)C), resuspended in 5 mL of 0.2 µm -filtered seawater. We inoculated approximately 40000 cells into 3 mL of 0.2 µm-filtered seawater (35 psu salinity determined by refractometer) in individual wells and allowed 1 h for the amoebae to attach to substrate at room temperature. We removed the seawater in wells and...
replaced it with 5 ml of 0.2 µm-filtered freshwater (≤ 1 psu once added to wells) for 1, 2, 4, 8, 15, 30, 60 and 120 min or with 5 ml of seawater for 0 min (10 sec) (30 wells in total). Each exposure duration was replicated three times. After each freshwater exposure, we reinstated seawater conditions by adding an extra 5 ml of double-strength seawater (70 psu) or filled seawater wells with an additional 5 ml of seawater. To assess gill-isolated *N. perurans* attachment and survival, we counted numbers of attached amoebae by light microscopy in a 5 mm² viewing arena at the well centre. Plates were slightly agitated to verify detachment. Assessments occurred at viewing times before and after exposures, as well as after a 1 h recovery period under seawater conditions.

2.2 Experiment 2 – Effect of freshwater exposure duration on *N. perurans* on AGD-affected gills

We lightly sedated (10 mg L⁻¹ Aqui-S) and transferred 80 post-smolt Atlantic salmon (mean ± S.E. weight = 107 ± 2 g; range = 83–140 g) from a holding tank into 4 x 280 L seawater (35 psu) experimental tanks (20 fish per tank at a density of 7.6 kg m⁻³). Experimental tanks were recirculating, constantly aerated, fitted with a protein skimmer and biofilter, and maintained at 15–16°C using a heater-chiller unit. After a 3-day acclimation period, we treated fish in each tank with antibiotics (590 mg L⁻¹ trimidine, active ingredients being sulfadimidine at 430 mg g⁻¹ and trimethoprim at 86 mg g⁻¹) for 30 min under no recirculation in response to a suspected bacterial skin infection. Then, fish were subjected to a 3 h AGD challenge using 1100 *N. perurans* cells L⁻¹, isolated as outlined in experiment 1, during ceased recirculation. After 4 days, during which time fish were expected to acquire light AGD infection levels (based on previous experience with the experimental system), we began daily freshwater treatment exposures of 3, 30 and 120 min or a procedural control seawater treatment of 3 min, in each of the 4 tanks which were for 6 days.

Freshwater treatments involved reducing the water volume in tanks to 80 L, then simultaneously emptying and filling tanks with dechlorinated mains supply freshwater held in reservoir tanks at 16°C using a pump (108 L min⁻¹) for 3 min by which point a salinity of ≤ 2 psu (measured by a refractometer) was achieved (Fig. 1). Freshwater treatment exposures began at this timepoint. Tanks were then refilled with freshwater for 1.5 min to slightly below their full capacity, so freshwater did not enter the biofilter, and salinities were ≤ 1 psu by this timepoint. To reinstate seawater conditions, tanks were
emptied to 80 L taking 1.5 min and ending freshwater treatment exposures, and then flushed with pumped seawater for 3 min, and then completely refilled with seawater to return them to 35 psu. The 3 min freshwater treatment involved emptying tanks (1:30 min) immediately after they had been filled with freshwater (1:30 min) (Fig. 1). We performed the same treatment procedures for a 3 min seawater procedural control treatment, except the freshwater-filling period was replaced with seawater-filling from reservoir tanks at 16°C, so that an identical amount of water exchange occurred.

One day after the final treatment, 10 fish from each tank were lethally sampled, and their 3rd right gill arch was removed and added to RNAlater for qPCR analysis to calculate numbers of host-attached *N. perurans*.

These steps were repeated in a second infection to replicate all treatments using another 80 post-smolt Atlantic salmon (mean ± S.E weight = 143 ± 3 g; range = 99–200 g; fish density of 10.2 kg m⁻³), and an identical AGD challenge of 1100 *N. perurans* cells L⁻¹.

*N. perurans* abundance determination via qPCR, arches were transferred to 1 mL lysis buffer (7.8M Urea, 0.5% SDS, 10mM Tris), briefly vortexed and left for 20 hours at -4°C. After brief centrifugation, 250 µL of sample solution was combined with 248 µL lysis buffer and 2 µL proteinase K (20 mg mL⁻¹) (Bioline, NSW, Australia) and incubated at 37°C for 30 min. After cooling at room temperature for 10 min, 250 µL of 7.5 M ammonium acetate was added and the sample was chilled on ice for 5 min to precipitate undigested proteins.

Samples were then centrifuged at 14,000 × g for 5 min. The supernatant was combined with 750 µL isopropanol, inverted 40 times and centrifuged again at 16,000 × g for 10 min. The nucleic acid pellet was rinsed twice with 70% ethanol, resuspended in 100 µL buffer (10 mM Tris, 0.05% Triton X100) and incubated for 15 min at 37°C. *N. perurans* abundance was determined through a real-time qPCR assay which distinguished an *N. perurans*-specific 18S rRNA gene sequence using a CFX Connect PCRT Detection System (Bio-Rad) (Wright, Nowak, Oppedal, Bridle & Dempster, 2015). Due to inhibition detected during a preliminary dilution series test, qPCR was performed on 1:20 diluted total nucleic acid extract. Amoebae numbers were estimated based on a copy number of 2880 per 1 *N. perurans* cell (Wright et al., 2015; Wright, Nowak, Oppedal, Bridle & Dempster, 2017a).

### 2.3 Statistical analysis

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Analyses were conducted in R software v.3.1.0 (R Core Team, 2016). For experiment 1, we used Welch two-sample t-tests (function t.test) to determine if differences existed in the number of attached gill-isolated *N. perurans* between each viewing time (before and immediately after exposures, and once a 1 h recovery period had elapsed) for each exposure type. Instances of no attached *N. perurans* in triplicate wells at a given viewing time and exposure type were not analysed. This resulted in no analyses at the 1 and 2 h freshwater treatments, because no attached amoebae were found after these exposures. Data were checked for normality using Shapiro-Wilk tests.

For experiment 2, we compared log-transformed *N. perurans* numbers per gill arch of individuals between treatments using linear models (function lm), including treatment (repeated 0 - seawater procedural control, 3, 30 and 120 min freshwater treatments) and infection (1 and 2) as fixed factors. From model sets including the null model (excluding the factor, treatment), and treatment + infection and treatment × infection models, the one minimising the Bayesian Information Criterion (BIC) by ≥ 2 compared to other models was selected (function BIC). For the best model, the significance of effects were tested using an anova test (function Anova, package car) (Fox & Weisberg, 2011). Because significant treatment × infection effects were identified, type III sums of squares were used. Following significant treatment × infection effects, least square mean comparisons were performed between treatments for each infection (function lsmeans, package lsmeans) (Lenth, 2016).

Data were checked to ensure residuals were well-behaved and that they were close to normally distributed (function plot).

### 3 RESULTS

#### 3.1 Experiment 1 – Effect of freshwater exposure duration on gill-isolated *N. perurans*

In the seawater procedural control, gill-isolated *N. perurans* remained attached and similar numbers of amoebae on the plastic substrate were found among viewing times (t ≤ |0.6|, p ≥ 0.6) (Figure 2 & 3). Following freshwater addition, amoebae quickly contracted their pseudopodia and progressively morphed from unattached flattened disc-shaped cells into more spherical floating pseudocysts (Figure 3). Spherical cells were initially crenated and then became smooth-surfaced, before finally rupturing and dying (Figure 3). For a 1 min freshwater exposure, numbers of attached *N. perurans* did not differ between before, after
and 1 h recovery viewing times (t ≤ |2.0|, p ≥ 0.2). However, after ≥ 2 min freshwater exposures, no *N. perurans* were observed on substrate (Figure 2). *N. perurans* subsequently resumed attachment to substrate after 1 h recovery for 2–30 min exposures. Numbers of attached *N. perurans* were similar between before and 1 h recovery viewing times for 4, 15 and 30 min freshwater exposures (t ≤ |2.0|, p ≥ 0.2), but reduced at 1 h recovery for 2 min (t = -6.1, p = 0.004) and 8 min (t = -4.2, p = 0.049). No live *N. perurans* were observed after 1 h recovery for the 1 and 2 h freshwater treatments (Figure 2).

3.2 Experiment 2 – Effect of freshwater exposure duration on *N. perurans* on AGD-affected gills

*N. perurans* numbers per gill arch on Atlantic salmon subjected to repeated seawater and freshwater treatments were influenced by treatment (F = 62.7, df = 1, p < 0.0001), infection (F = 16.2, df = 3, p < 0.0001) and treatment × infection interaction effects (F = 6.7, df = 1, p = 0.0004) (Table 1, Figure 4). Infection and treatment × infection interactions arose from lower numbers of *N. perurans* per gill arch in infection 1 (mean of 12.8) compared to 2 (mean of 299.1) (Figure 4). In infection 1, *N. perurans* numbers on gills were similar between repeated seawater procedural control and freshwater 3 min treatments (means of 42.3 vs 8.8, p = 0.4), while no cells were detected on gills exposed to repeated freshwater 30 and 120 min treatments. For infection 2, *N. perurans* numbers on gills were greater for the repeated seawater procedural control (mean of 814.5) than repeated freshwater 3 (mean of 361.9) (p = 0.02), 30 (mean of 20.0) (p < 0.0001) and 120 min treatments (mean of 0.1) (p < 0.0001). Similarly, presence of *N. perurans* per gill arch was comparably high between the repeated seawater procedural control (95%) and freshwater 3 min (100%) treatments, and low for repeated freshwater 30 min (25%) and 120 min treatments (10%) (Figure 4).

4 DISCUSSION

We found ≥ 2 min exposures completely lifted amoebae populations from plastic substrate. However, the freshwater-challenged *N. perurans* survived as pseudocysts and reattached to the plastic once seawater conditions were restored before lysis occurred at ≥ 60 min exposures. An *in vivo* experiment revealed that repeated freshwater exposures of 30
minutes that are sub-lethal to gill-isolated *N. perurans* consistently reduced their presence on AGD-affected Atlantic salmon to levels comparable to repeated lethal 120 min freshwater exposures – a duration of freshwater bathing used against AGD by the salmon farming industry. This suggests that detachment and formation of pseudocyst by *N. perurans*, rather than mortality, may play a role in reducing *N. perurans* populations on gills in freshwater treatments. We observed inconsistent reductions of amoebae on gills from brief repeated sub-lethal 3 min exposures that detach gill-isolated *N. perurans* on plastic. This suggests that host-dependent mechanisms (for example presence of gill mucus) partially protect them from freshwater exposures in the short-term. Based on our results, repeated freshwater exposures for durations targeting cell detachment rather than death, could replace single lethal exposure durations for AGD treatment.

**4.1 Gill-isolated *N. perurans* in freshwater**

We corroborated recent observations of rapid detachment by gill-isolated *N. perurans* on plastic once subjected to freshwater and transition to a floating pseudocyst form (Lima et al., 2017). Cowie and Hannah (2006) also found a range of naked marine amoebae changed from attached to spherical floating forms once a low salinity threshold was reached. They postulated this was either a behavioural response to unfavourable conditions or reducing their surface area to volume ratio and intrinsic water uptake rate to lower the risk of lysing or a shift to the spherical floating form resulted from interference with attachment and locomotion processes (involving pseudopodia) once intracellular ion concentrations fell. If salinity reduction was a single shock rather than prolonged over gradual steps amoebae populations could be completely detached by higher salinities (Cowie and Hannah 2006).

Our fine-temporal scale data demonstrated that this behavioural- or physiological-driven detachment response to a single osmotic shock can occur completely after ≥ 2 minutes for gill-isolated *N. perurans* populations attached to plastic following freshwater immersion.

Our results confirm that after detaching from substrate, *N. perurans* can survive as floating pseudocysts and successfully reattach again once salinity conditions improve as shown before (Lima et al., 2017). *N. perurans* osmoregulate in hypo-osmotic conditions using contractile vacuoles to expel water flowing into the cell (Lima, Taylor & Cook, 2016). In this state without growth or reproduction, some species of naked marine amoebae remain
viable even after one week at $2\,\text{g}\,\text{L}^{-1}$ if transferred to $32\,\text{g}\,\text{L}^{-1}$ (Cowie & Hannah, 2006). Our findings suggest that *N. perurans* do not survive as pseudocysts for $\geq 1\,\text{h}$ in deionised water under *in vitro* conditions ($\leq 1\,\text{g}\,\text{L}^{-1}$). However, others have observed high recovery rates at this exposure duration but reduced recovery at 2–3 h (Lima et al., 2017; Powell & Clark, 2003). The disparity in freshwater survival times between studies could relate to different culture conditions, amoeba strains, and survival assessment methods. There is consensus between studies, though, that gill-isolated *N. perurans* have high recovery rates *in vitro* after short 0–30 min freshwater exposures (Lima et al., 2017; Powell & Clark, 2003).

4.2 *N. perurans* on AGD-affected gills in freshwater

Repeated 30 min freshwater exposures, which are sub-lethal and dislodge gill-isolated *N. perurans* in a single treatment, were sufficient to reduce or clear populations on AGD-affected Atlantic salmon gills. The reduction of *N. perurans* at 30 min freshwater exposures suggests that detachment of *N. perurans*, as they form pseudocysts (Lima et al., 2017), is partially involved in reductions of populations on gills during freshwater treatments. However, other processes such as mucus hydration and viscosity reduction, fragmentation of AGD lesions and subsequent removal of host cells, and amoeba-gill epithelia interactions are also likely to influence the ejection rates of *N. perurans* from gills (Clark et al., 2003; Parsons et al., 2001; Roberts & Powell, 2003; Roberts & Powell, 2005; Wiik-Nielsen, Mo, Kolstad, Mohammad, Hytterød & Powell, 2016). It is also possible that *N. perurans* were eventually killed by the repeated 30 min freshwater exposures. However, *N. perurans* are capable of replicating in 24 h (P. Crosbie pers. obs.), and so newly replicated and unchallenged amoebae may have been present for each repetition of the daily treatments. Repeated 120 min freshwater exposures that significantly reduce recovery rates of gill-isolated *N. perurans* (Lima et al., 2017), were comparable in efficacy to 30 min repeated exposures. Interestingly, some amoebae were still detected after 120 min treatments. These remaining cells may represent a fraction of the population that tolerated repeated extended freshwater exposures as in Lima et al. (2017), or were amoebae from the biofilter in the recirculating tanks causing re-infections.
*N. perurans* populations on gills were inconsistently lowered by repeated 3 min freshwater exposures that cause detachment but are survived by gill-isolated *N. perurans in vitro* in a single event. This inconsistency may have partly originated from infection intensity, with difficulties in detecting differences between the low *N. perurans* numbers on gills for the repeated 3 min freshwater and seawater exposures in infection 1. However, cell-to-cell interactions, mucus, AGD lesion development and gill surfaces may also help *N. perurans* on gills to remain attached longer than gill-isolated cells on plastic in freshwater. Instead, time until *N. perurans* detachment from gills in freshwater may be partially related to how quickly mucus and cell debris is released. Our experiment suggested that the combination of *N. perurans* detachment from gills and mucus and debris shedding following freshwater exposure acts consistently over ≥ 3 min time scales. The exact host-dependent mechanisms potentially used by *N. perurans* for attachment to gills in freshwater require further investigation.

### 4.3 Implications for amoebic gill disease management

Our results indicate that freshwater bathing could be formulated differently to current industry practices. Present-day freshwater bathing exposes fish to ≤ 3 g L$^{-1}$ water for 2–4 h (Oldham et al., 2016), and are based on freshwater durations that are lethal to gill-isolated *N. perurans* (Powell & Clark, 2003). However, we showed here that using repeated 30 min sub-lethal freshwater treatments designed to detach *N. perurans* could be an alternative strategy.

While repeated exposures are difficult to achieve using traditional bathing practices such as a tarpaulin lined cage or well boat, they are possible with recently developed *in situ* bathing methods. A freshwater layer within a surface enclosure in a ‘snorkel’ cage reduced an outbreak of amoebic gill disease in commercial sea cages for Atlantic salmon in Norway (Wright et al., 2017b). Salmon likely entered the enclosure as it was the only place within the snorkel cage that they could reach the surface and refill their open swim bladder by swallowing air at the surface for buoyancy control (Dempster et al., 2011). Use of the freshwater surface layer coincided with reductions in amoebic gill disease levels (Wright et al., 2017b), which suggest that fish were utilising the layer for > 3 min daily exposures for swim bladder reinflation and due to other motivational factors (Oppedal et al., 2011), or
that daily exposures of ≤ 3 min were over longer periods than the current trial. This commercial example may open the way for implementation of similar strategies for other AGD-affected marine fish species where freshwater exposures are practical.

Changing freshwater treatment strategies from using single long lethal treatments to repeated short sub-lethal treatments could reshape the trajectory of freshwater tolerance evolution in *N. perurans* populations. Current use of freshwater treatments against AGD aimed at killing amoebae may be responsible for the removal of freshwater sensitive strains and retention of freshwater tolerant ones in *N. perurans* populations; causing freshwater tolerance evolution. This could be partially linked to increasing freshwater treatment frequency in SE Tasmania where recurrent AGD outbreaks occur (Rodger, 2014). Using a moderate approach of repeated shorter freshwater bath treatments aimed at detaching rather than killing amoebae on gills, may lead to reduced selection pressure for freshwater tolerant strains (some evidence from drug resistance evolution studies) (Kouyos et al., 2014). However, because freshwater tolerant *N. perurans* strains potentially outcompete susceptible ones under moderate sub-lethal exposures, which is how drug resistant bacteria can evolve from sub-lethal drug doses (Colijn & Cohen, 2015), current rates of freshwater tolerance evolution may remain unchanged or accelerate. In addition, Hughes and Andersson (2017) acknowledged that mutation rates, performance of resistance mechanisms, fitness of resistant relative to susceptible strains, epistatic interactions, compensatory evolution and population bottlenecks can influence drug resistance evolution. Given the complexities likely involved in freshwater tolerance evolution by *N. perurans*, it is difficult to predict the effects of moderate vs aggressive approaches to freshwater treatment of AGD before quantitative evidence is at hand.

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Wright, D.W., Nowak, B., Oppedal, F., Bridle, A. & Dempster, T. (2017a) Free-living *Neoparamoeba perurans* depth distribution is mostly uniform in salmon cages, but

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**Figure legends**

FIGURE 1 Examples of salinity conditions during each treatment type (seawater 3 min and freshwater 3, 30 and 120 min) repeated daily over 6 days in tanks holding AGD-affected Atlantic salmon.

FIGURE 2 Mean (± S.E.) of attached gill-isolated *N. perurans* numbers at each viewing time (before - grey bars, after, and at 1 h recovery times) and exposure type (seawater for 0 min, or freshwater for 0, 1, 2, 4, 8, 15, 30, 60 and 120 min). Differences in attached *N. perurans* numbers between viewing times (brackets and asterisks indicate statistically different groups based on t-tests, p < 0.05) are displayed. Note that tests were not performed if no amoebae were observed in triplicate wells at a viewing time.

FIGURE 3 Images display gill-isolated *N. perurans* under microscopy (100× magnification) in a) floating forms prior to attachment in seawater, b) attached forms on plastic substrate in seawater, c) disc-shaped pseudocyst forms detached from substrate at 5 min in freshwater, d) crenated spherical pseudocyst forms following 30 min in freshwater, and e) smooth spherical pseudocyst forms or lysed cells at 50 min of freshwater exposure.

FIGURE 4 *N. perurans* per gill arch on post-smolt Atlantic salmon exposed to an AGD challenge and repeated treatments (seawater 3 min shown here as freshwater 0 min and freshwater 3, 30 and 120 min) are displayed in terms of cell numbers on a log scale (top, box plots) and presence (bottom, bars). Grey boxes and bars indicate *N. perurans* cell numbers and presence for individuals exposed to only seawater. The zero ticks are slightly raised in the top panels, so circles representing zero *N. perurans* per gill arch are clearly visible. Least square mean differences (p < 0.05) in treatments within each infection are displayed. The zero ticks are slightly raised in the top panels, so circles representing zero
*N. perurans* per gill arch are clearly visible. Differences in *N. perurans* numbers on gills between treatments within each infection are displayed (brackets and asterisk indicate statistically different groups based on least square means comparisons, p < 0.05). Note that differences are not presented if amoebae cells were not detected on fish at a treatment within an infection.

Wright et al. FIGURE 1

![Graph showing salinity over time for different conditions](image)

Wright et al. FIGURE 2

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### Table 1 Comparisons between linear models including treatment and infection as factors in explaining log-transformed *N. perurans* on gills using the Bayesian Information Criterion (BIC).

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**Table legends**

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