Protist, Vol. 170, 153–167, April 2019
http://www.elsevier.de/protis

Protist

ORIGINAL PAPER

Survival and Growth in vitro of *Paramoeba perurans* Populations Cultured Under Different Salinities and Temperatures

Catherine Collins[1], **Malcolm Hall**[1], **Mark J. Fordyce, and Patricia White**

Marine Scotland, 375 Victoria Road, Aberdeen AB11 9DB, United Kingdom

Submitted April 9, 2018; Accepted November 5, 2018
Monitoring Editor: C. Graham Clark

Growth rates of *Paramoeba perurans* cultures under different temperature and salinity conditions were investigated in vitro over a 15 day period. Optimal population growth, under the experimental conditions, was observed at 15 °C and a salinity of 35‰, with amoebae populations doubling every 14 h. Positive *P. perurans* populations growth was observed at 15 °C between salinities of above 20‰ and 50‰, and at 8 °C, 11 °C and 18 °C at salinities between 25‰ and 50‰, 50‰ being the maximum salinity tested. Amoebae numbers were sustained at 4 °C. Therefore, lower temperature and salinity thresholds for *P. perurans* population growth lie between 4 to 8 °C, and salinities of 20 to 25‰, respectively. Upper limits were not determined in this study. The populations remained relatively stable at 4 °C and 2 °C at permissive salinities with respect to numbers of viable amoebae over the 15 day exposure period.

Key words: *Paramoeba perurans*; in vitro; population growth; salinity; temperature.

Introduction

Amoebic gill disease (AGD) caused by the amoeboid parasite *Paramoeba perurans* is one of the most important parasitic diseases for the salmon aquaculture industry in terms of prevalence, fish loss and treatment costs (Rodger 2014; Shinn et al. 2015 and references therein), and has also been reported as causing disease in other fish species (Crosbie et al. 2010; Haugland et al. 2017; Karlsbakk et al. 2013; Kim et al. 2017). The disease has been recorded globally (Bustos et al. 2011; Crosbie et al. 2010; ICES 2015; Kent et al. 1988; Mouton et al. 2013; Rodger and McArdle 1996; Steinum et al. 2008; Young et al. 2008b), and has been particularly problematic for the Tasmanian salmon industry where treatment has been reported to add 14–20% in production costs (Munday et al. 2001; Nowak 2012). Incidences of the disease had been sporadic in northern Europe (Rodger and McArdle 1996; Steinum et al. 2008) until relatively recently but have increased since 2011/2012 (Oldham et al. 2016; Rodger 2014) and now represent a significant health issue, not only directly, but in potentially compromising the ability to safely treat for sea lice (*Lepeophtheirus salmonis*) infections, another major parasitic challenge for salmon aquaculture (Pert et al. 2015).

---

[1] Corresponding authors.
e-mails Catherine.Collins@gov.scot (C. Collins), Malcolm.Hall@gov.scot (M. Hall).

https://doi.org/10.1016/j.protis.2018.11.003
1434-4610/

One of the fundamental aspects of developing approaches to management and control of disease is an understanding of the pathogen biology and its interaction with the local environment and host. Surprisingly little is known of *P. perurans* biology and environmental interactions. There have been limited published experimental studies on the effect of salinity and temperature on survival and population growth of *Paramoeba perurans*. This is largely due to the fact that *P. perurans* was only identified as the causative agent of AGD in 2007 (Young et al. 2007) and the ability to culture it in vitro was only achieved relatively recently (Crosbie et al. 2012). The latter has increased the scope for experimental work to study the parasite. Prior to this the causative agent of AGD was thought to be *Paramoeba pemaquidensis* (Kent et al. 1988) and some studies had been performed to understand basic biological traits (Martin 1985) and its distribution in the environment (Douglas-Helders et al. 2003, 2005; Tan et al. 2002). Recent studies on *P. perurans* itself, outwith interaction with host, have investigated presence of *P. perurans* in the water column, on various structures, macrofauna and sediment on salmon farms, and in wild fish (Bridle et al. 2010; Hellebø et al. 2017; Nowak et al. 2010; Stagg et al. 2015; Wright et al. 2015).

Given that *P. perurans* is a facultative marine parasite, one of the most basic questions in understanding the infection dynamics and epidemiology of AGD is, what is the potential for propagation and survival of *P. perurans* over the range of temperatures and salinities encountered in its marine environment? This can inform on conditions giving rise to increased parasite numbers and associated risk of disease outbreaks, and on the ability of the parasite to survive in different locations and through seasonal changes. It can also inform on the potential of possible vectors to harbour or transport the parasite. In terms of AGD itself, there are a number of observational studies which have highlighted its seasonality and the environmental factors which favour disease outbreaks, namely salinities of greater than 32‰ and temperatures of 12 °C and over, although disease has been reported at lower salinities and temperatures (Oldham et al. 2016; Steinum et al. 2008). Favourable conditions for the disease may reflect an interaction between both amoebae and host responses. Though there have also been a number of studies on the survival of *Paramoeba* following freshwater exposure, in the context of freshwater as a treatment for AGD (Clark et al. 2003; Green et al. 2005; Lima et al. 2016; Powell and Clark 2003; Roberts and Powell 2003), very limited information is available on the environmental conditions supporting *P. perurans*, since its confirmation as the causative agent of AGD.

The objective of the current study was to obtain data on the range of temperatures and salinities under which *P. perurans* survives, and changes at a population level.

The study was performed with in vitro cultures of *P. perurans* and not in a host environment.

Results

Persistence of Amoebae at Different Temperature and Salinity Combinations at One Day Post Exposure

Results are presented in Figure 1. Observed and modelled values indicate an increase in amoebae number from low numbers at low salinities to numbers similar to those initially added at intermediate and high salinities for all temperatures. The number of amoebae initially added to each flask is not known precisely and relatively small deviations in values should not be interpreted as being biologically important. No live amoebae were observed at salinity 0‰ for all temperatures. The 1.43% sample volume corresponds to 300 seeded amoebae and the lack of live amoebae corresponds, assuming the probability mass function of the binomial distribution, to a 95% chance that freshwater kills not less than 99% of amoebae within one day (earlier time points not investigated). However, the modelled survival of some amoebae at 0‰ for all temperatures except 8 °C, where values were effectively 0, indicated that some amoebae may be capable of persisting in freshwater for at least one day. Additionally viable amoebae were observed at salinity 3‰ for all temperatures, and included stained suspended cells at 2, 4, 11, and 15 °C confirming that amoebae persist for at least one day at low but non-zero salinities. In summary at the very least a high proportion of amoebae do not survive 0‰ salinity for one day across a range of temperatures, although some survival cannot be discounted.

Persistence of Amoebae at Different Temperature and Salinity Combinations 15 days Post Exposure

Results are presented in Figure 2. Attached, rounded amoebae were observed in some replicates at 0‰ for 8 and 18 °C, indicating that amoebae may possibly persist in freshwater for at

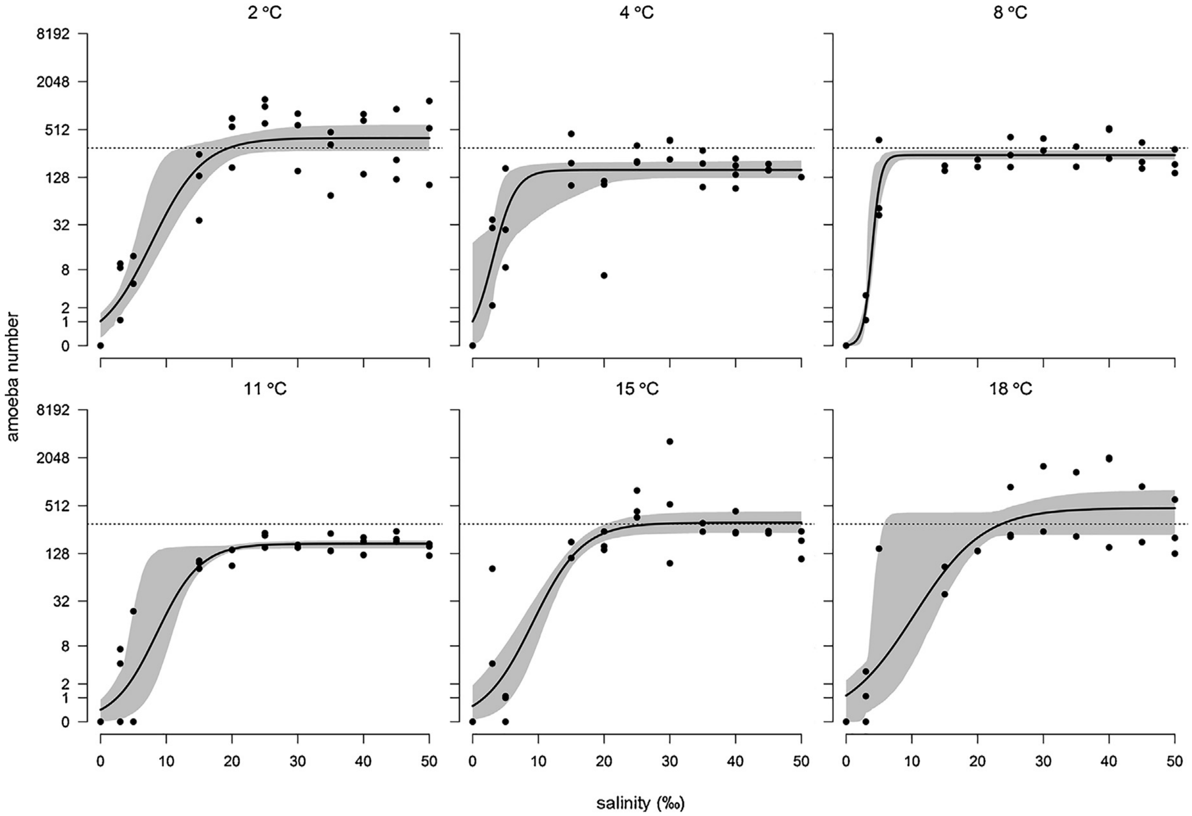

Figure 1. Number of viable amoebae on day 1 following inoculation at different temperatures and salinities for a 1.4% sample of underlay and overlay. Observations are indicated by points (●), predicted amoeba number by a continuous line (—) and 95% CI by dark grey shading. The approximate number of inoculated amoebae is indicated by a dotted line (···).

Table 1. Approximate salinity at intersect of logistic curves for day 1 and 15.

Temperature (°C)	Salinity and 95% CI (‰)
2	No intersect
4	31 (27-no intersect)
8	22 (21–23)
11	20 (18–22)
15	20 (19–20)
18	22 (20–23)

least 15 days. While consistent with a low level of survival the possibility that these are non-viable cannot be discounted. Predicted amoeba numbers at salinities of less than 20‰ were lower on day 15 than on day 1 for all temperatures, indicating a salinity threshold of not less than 20‰ for population maintenance over the 15 day period. Salinities at the intersect of predicted amoeba numbers on day 1 and 15 range from 20 to 31‰ (Table 1). Predicted amoeba numbers above 20-22‰, were higher on day 15 than on day 1 at 8, 11, 15 and 18 °C and above 31‰ equivalent at 4 °C (Table 1). This indicates that the minimum permissive temperature for colony-growth, at permissive salinities, occurs from 4 to 8 °C. In summary, amoeba population declined over 15 days at salinities below 20‰ at all temperatures, although some survival at 0‰ salinity cannot be discounted.

Persistence of amoebae over time at different temperature and salinity combinations

Results are presented in Figure 3. Some amoebae were observed at a salinity of 0‰ at intermediate timepoints between 1 and 15 days post-inoculation for all temperatures, indicating that amoebae may persist in freshwater greater than one day. The majority of these (66 of 68 amoebae) were attached on which vital staining was not carried out. Two suspended cells, stained with vital dye, on day 12 were observed, one at 8 °C and another at 18 °C. Again, “0” counts were obtained intermixed with positive counts on consecutive days, consistent

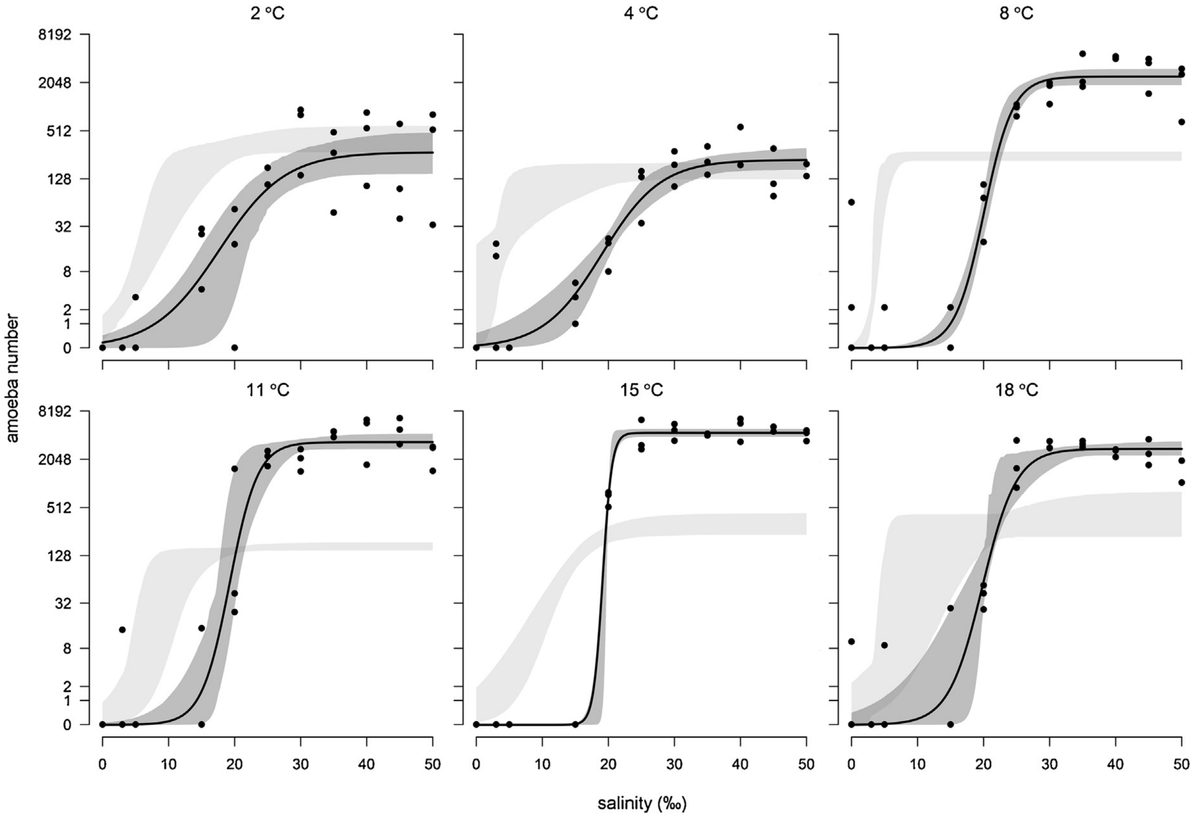

Figure 2. Number of viable amoebae on day 15 following inoculation at different temperatures and salinities for a 1.4% sample of underlay and overlay. Observations are indicated by points (●), predicted amoeba number by a continuous line (—) and 95% CI by dark grey shading. The 95% CI of amoeba number on day 1 (as shown in Fig. 1) is indicated by light grey shading.

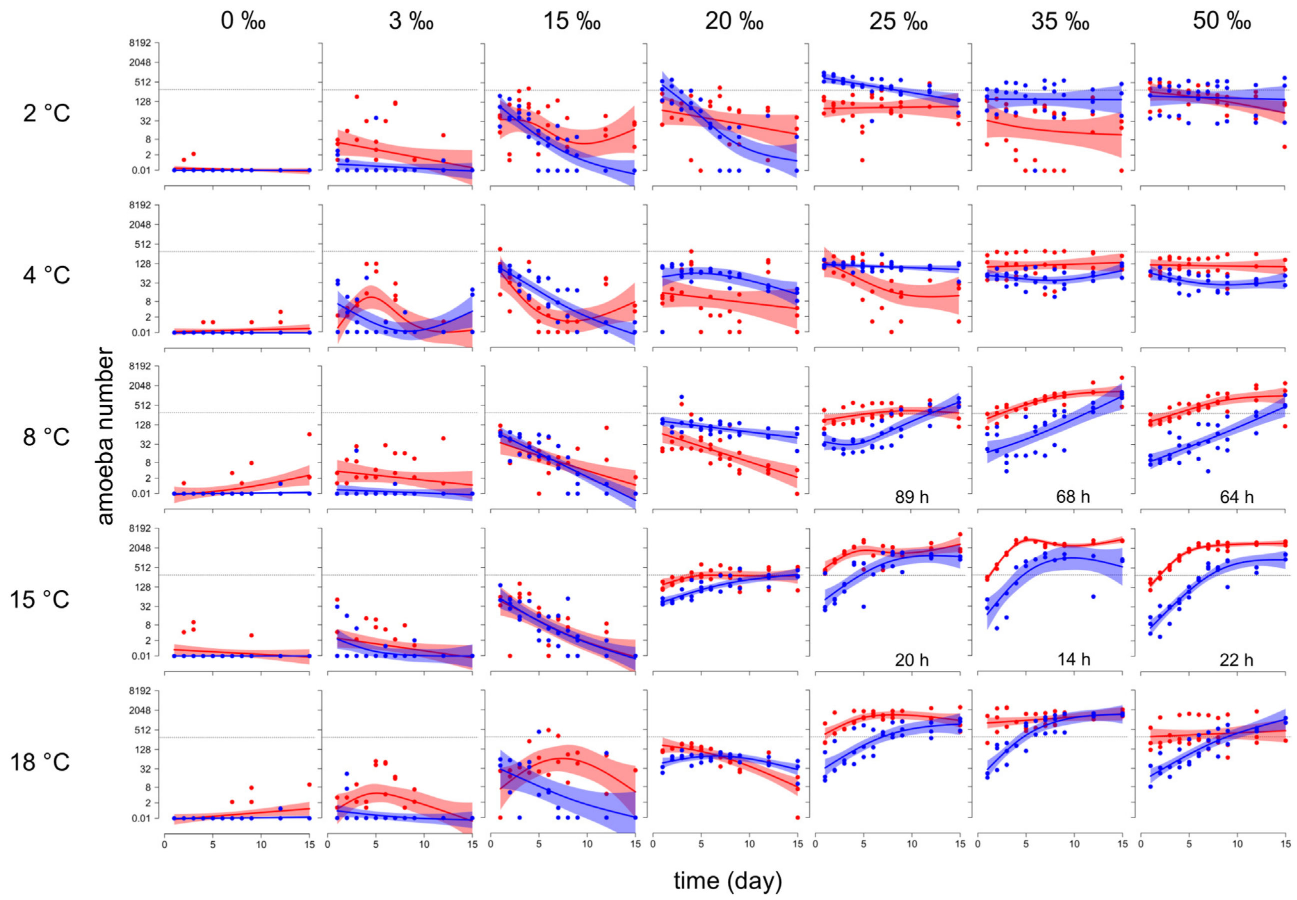

Figure 3. Number of viable attached and suspended amoebae between days 1 and 15 following inoculation at different temperatures and salinities for a 1.4% sample of underlay and overlay. Observations are indicated by dots (red dots [in greyscale version ●] and blue dots [in greyscale version ○] for attached and suspended respectively), predicted amoeba number by lines (—— and ---- respectively) and 95% CI by red and blue shading (in greyscale version ■ , □ respectively). The approximate number of inoculated amoebae at day 0 is indicated by a light horizontal dotted line (···).

with sampling variation around a low number of counts. Predicted lower 95% CI of amoeba number at salinity of 20‰, for suspended and/or attached amoebae, are considerably greater than 0 for all temperatures (Fig. 3), indicating that salinity of approximately 20‰ facilitates the persistence of amoeba for at least 15 days, at the very least.

At salinities and temperatures greater than or equal to 25‰ and greater than 4 °C, the number of attached amoebae was greater than those of amoebae in suspension over all or most of the 15 day period monitored. Increases in suspended amoebae numbers started from early in the 15 day period, indicating that a threshold level of attached amoebae before "shedding" of amoebae into suspension was not required. This is assuming that amoebae do not divide in suspension, increasing in numbers over time under permissive conditions.

At salinities and temperatures not permissive of population growth, numbers of attached amoebae were generally lower compared to amoebae in suspension, at day 1 post seeding. These differences were evaluated on absence of overlap between the 95% CI of attached and suspended amoebae counts (Fig. 3, Table 3). The observed pattern indicates that non-permissive temperatures and salinities may adversely affect amoebae attachment.

Growth Rates of *P. perurans* Population Under Different Temperature and Salinity Combinations

Results are presented in Table 2. Doubling times at maximum growth are given only for temperature and salinity combinations at which amoeba numbers increased over time and for which a majority of bootstrap samples generated an estimate. Estimates of doubling times range from 89 (24–143) hours at 8 °C and 25‰ to 14 (11–17) hours at 15 °C and 35‰. The 95% CI are on occasion considerable indicating uncertainty of the true value. Differences in doubling time appears to be influenced to a greater extent by temperature compared to salinity.

Discussion

Increasing salinities and temperatures, and salinities of greater than 32‰ in particular, have been highlighted as a risk factor for amoebic gill disease (AGD) outbreaks (Adams and Nowak 2003; Bustos et al. 2011; Clark and Nowak 1999; Douglas-Helders et al. 2001; Douglas-Helders et al. 2003, 2005; Mouton et al. 2013; Steinum et al. 2008). In recent years AGD has been reported all year around in Tasmania, and globally at temperatures as low as 7 °C, perhaps indicating that temperature, as an environmental constraint, may be less important than previously thought (Oldham et al. 2016). However, even small increases in temperature, potentially associated with climate change, may be sufficient for populations of amoebae to attain thresholds for survival and/or growth that significantly impact on the disease dynamics.

The current analysis focuses on the persistence of amoebae at the population level. This is because inferences regarding persistence from this laboratory experiment are more likely to be applicable to aquaculture field conditions than those concerning optimum population growth. On occasion however, some observations regarding amoebae colony-growth have been made where inference from in-vitro to the field was felt to be justified. Inferences relate to the population level because persistence could arise from either the long-term viability of individual amoebae or the continuing replacement, without colony-growth, of amoebae and these aspects were not investigated.

Paramoeba perurans in the current study displayed a minimum threshold salinity for population growth (as opposed to the threshold for any individual amoeba to divide) in vitro of between 20–25‰ at tested temperatures permissive of growth. In relation to other *Paramoeba* species for which data exist, the salinity threshold for *P. perurans* population growth appears to be higher than that of 8 to 9‰ at 15 °C observed, in vitro, for *Paramoeba pemaquidensis* (Kent et al. 1988), a causative agent of mortality in American lobster (Mullen et al. 2005).

In terms of temperature thresholds, *P. perurans* positive population growth was only observed at temperatures of 8 °C or higher, although given that the population was sustained at 4 °C, growth at less than 8 °C cannot be discounted (*P. perurans* population growth at temperatures between 4 and 8 °C was not investigated). Amoebae survived at 2 to 4 °C over the 15 day period. Similarly, the minimum temperature required for positive population growth for *Paramoeba invadens* (causative agent of wasting disease in the green sea urchin *Strongylocentrotus droebachiensis*) was predicted to occur around 7–8 °C, at a salinity of ∼35‰ (Fig. 3, Buchwald et al. 2015), and *P. invadens* populations did not grow at 5 °C and below (Buchwald et al. 2015; Jellett and Scheibling 1988), although amoebae survived for significant amounts of time at

Table 2. Minimum *Paramoeba perurans* doubling-times in hours for different temperature and salinity combinations with 95% CI in brackets. Estimates are only provided for combinations at which amoeba numbers increased over time and for which a majority (>50%) of bootstrap samples generated an estimate. Additional estimates of minimum doubling times not included in the table are 97 hrs (23–187) for 4 °C, 40 ‰, and 48 hrs (12–89) for 18 °C, 30‰.

Salinity (‰)	Temperature (°C)		
	8	11	15
25	89 (24–143)	47 (39–56)	20 (14–27)
30	62 (42–87)	44 (38–50)	18 (8–192)
35	68 (47–88)	36 (31–41)	14 (11–17)
40	58 (43–75)	35 (16–48)	15 (11–20)
45	57 (32–80)	46 (32–59)	16 (15–18)
50	64 (39–87)	60 (48–70)	22 (19–26)

Table 3. Status of amoebae in relation to attachment to malt yeast agar underlay, or suspended in seawater overlay, on day 1 post seeding of culture flasks, under different salinities and temperatures. A: higher numbers attached, S: higher numbers in suspension. –: no apparent difference.

Temperature (°C)	Salinity (‰)						
	0	3	15	20	25	35	50
2	–	A	–	–	S	–	–
4	–	–	–	–	–	–	–
8	–	A	–	–	A	A	A
15		–	–	A	A	A	A
18	–	–	–	A	A	A	A

these temperatures and exhibited recovery of population growth dependent on duration of exposure to the lower temperatures. A minimum permissible growth temperature has not been reported for *P. pemaquidensis*, although slow amoebae population growth was reported at 5 °C (at 25‰) (Kent et al. 1988), lower than reported for *P. invadens*, and potentially *P. perurans*. However, figure 8 in Kent et al. (1988) indicates positive growth at the population level, at 96 h, only at 10 °C and above. An upper survival temperature was not determined for *P. perurans* in this current experiment, although interestingly, a reduction in final population size at 18 °C compared to 11 °C and 15 °C, also observed by Crosbie et al. (2014) although not consistently across their different experiments, could be interpreted as indicative of environmental stress.

These observations of reduced *P. perurans* growth in vitro at 18 °C appear to conflict with previous field studies (Clark and Nowak 1999), or industry observations, which reported an increased prevalence and severity of AGD associated with higher temperatures. A conflict assumes that disease severity is associated with amoebae load (Collins et al. 2017; Downes et al. 2017); although not all studies would concur with this (Fringuelli et al. 2012). It should be kept in mind that these studies used differently prepared material and approaches, with differences in temperature and times post infection when gills sampled. It may be that temperatures above a threshold detrimentally affect host resilience, rather than increase amoeba numbers, and so promote AGD development (Oldham et al. 2016). Alternatively, conditions at 18 °C within an enclosed in vitro system may not support continued *P. perurans* population growth, perhaps due to a build up of toxic metabolites. Finally it may be that field investigations or industry observations involve variation around a nominally recorded temperature and that reduced population growth at suboptimal temperatures (e.g. 18 °C) is more than compensated for by increased population growth at optimal temperatures (e.g. 15 °C) if these occur, leading to an overall increased occurrence and severity of AGD. In summary, the results of this laboratory experiment and previous field studies and industry observations are not necessarily inconsistent.

P. perurans optimal growth rate in vitro was observed at 35‰ and at 15 °C with a doubling in *P. perurans* numbers estimated every 14 (11–17) hours. Doubling times of 16–19.4 h, 73.4 h and 58.4–91.2 h were observed at 15–20 °C, 12 °C and 10 °C (at 35–36‰) respectively for *P. invadens* (Buchwald et al. 2015; Jellett and Scheibling 1988). P*. pemaquidensis* had a doubling time of approximately 17.8 h, 27.7 h and 107 h at 15 °C/15‰, 15 °C/25‰ and 10 °C/25‰, respectively (estimated from figures 7 and 8 in Kent et al. 1988). The lowest doubling period of 14 h for *P. perurans* in the current study is similar to that found in the other *Paramoeba* species studied, under the growth conditions tested. Of note is the optimal growth of *P. pemaquidensis* (96 h post seeding) at the lower salinity of 15‰ (at 15 °C), reflecting the lower salinity threshold of 8–9‰ required for population growth of this species.

Based on the doubling times for *P. perurans* in the current study, and estimates for *P. pemaquidensis* from Kent et al. (1988), the results indicate that both salinity and temperature can affect growth rate, but that, at permissive growth ranges for both parameters, temperature appears to have a greater impact on the rate of population growth. Salinity may be more important in predicting survival (here and Douglas-Helders et al. 2005). Having said this, optimal conditions for disease outbreak or severity, and optimal in vitro conditions for amoebae population growth, need not necessarily be the same. Conditions also affecting the host, or host-parasite interaction should be taken into account, as suggested in Oldham et al. (2016).

In terms of understanding the epidemiology of disease outbreaks, the in vitro growth studies on *P. invadens* (Buchwald et al. 2015; Jellett and Scheibling 1988) have helped highlight conditions for its survival and potential for overwintering in the coastal waters of Nova Scotia (Buchwald et al. 2015; Feehan et al. 2016; Scheibling and Lauzon-Guay 2010). Temperatures range between 4–9 °C and 15–18 °C for Scottish west coast surface waters (http://www.gov.scot/Resource/Doc/345830/0115122.pdf; https://www.seatemperature.org/europe/). Unfortunately little data are available from deeper coastal waters. These temperatures may allow long term survival of *P. perurans* in the environment at all times of the year in Scotland, based on their in vitro survival and population growth results. Greater resolution of population growth potential between 4 °C and 8 °C is required to see if minor temperature changes within this range could signify biologically significant changes in growth. The situation in Norwegian coastal waters may differ, in that in some sites temperatures towards the surface of the water column are likely to fall below 4 °C during the winter months (Sætre and Ljøen 1972; Sætre et al. 2003); with ranges of 0.47–19.72 °C, 3.79–16.34 °C, 3.89–10.87 °C, and 5.66–9.8 °C, 6.99–9.61 °C, 4.80–8.33 °C at 5 m and 150 m respectively for averaged monthly temperatures between 2006–2014, for monitoring sites Lista, Bud and Ingoy (http://www.imr.no/forskning/forskningsdata/stasjoner/view?station=Ytre%20Utsira; https://www.seatemperature.org/europe/). This may result in a corresponding potential fall off in the local amoebae load. Higher winter temperatures in a given year may predispose sites to earlier outbreaks the following spring if amoebae are present on site, or present at sites from which amoebae are dispersed.

Environmental parameter thresholds for amoebae population growth and survival can also inform on natural habitats/reservoirs for amoebae species (Scheibling and Lauzon-Guay 2010) though it may also be possible that cyst-like forms can survive for substantial time periods and recover under suitable conditions (Lima et al. 2016; Scheibling and Lauzon-Guay 2010).

The source of *P. perurans* causing new or initial infection has still to be determined e.g. local reservoirs, from coastal sites, or from sources originating offshore. Some amoebae species are able to survive and grow under extreme ranges of salinity, with their natural environment dictating to some extent the range of salinity tolerances observed (Hauer and Rogerson 2005). With respect to *Paramoeba* species, *P. pemaquidensis* and *P. perurans* have minimum salinity thresholds for population growth of approximately 8–9‰ and 20–25‰, respectively. The higher salinity required by *P. perurans* for population growth may indicate that it is more adapted to a natural habitat not subjected to high freshwater input – i.e. offshore/deeper water sites. Circumstantial evidence to support this possibility is that whereas *P. pemadquidensis* has readily been described from coastal sediment and water samples over a wide geographical area (Cann and Page 1982; Page 1983), *P. perurans* or AGD for the most part has been found in samples associated with aquaculture sites with no or very low prevalences detected in wild fish surveys (Bridle et al. 2010; Douglas-Helders et al. 2002; Hellebø et al. 2017; Stagg et al. 2015). An alternative explanation is that *P. perurans* only reaches detectable environmental levels when growing on an optimal substrate such as host gills in an aquaculture environment with concentrated host numbers. *Paramoeba perurans* has been reported from deep water in Norway, and it has been suggested that breakdown of stratification in autumn and mixing of surface and deeper waters may be linked to higher incidence of AGD in Norway during autumn months (VKM report 2014). The in vitro results show that *P. perurans* populations can grow at 20–25‰, so short-term exposure to suboptimal salinities may not be an issue. The longer-term effect of exposure to low salinities on the ability to survive and to subsequently infect fish should be investigated.

There is some indication that an interaction may exist between temperature and salinity in determining the tolerance limits permissive of *P. perurans* population growth (as opposed to optimal growth). *Paramoeba perurans* population growth at sub optimal temperatures may require higher salinities above the salinity threshold of 20–25‰. Though not statistically supported as positive population growth, the equivalence with day 1 in amoebae numbers at 4 °C at end of experiment was only seen at salinities of 31‰ and above, whereas population growth at 8 °C and above occurred at 20–25‰ °C. This interaction between temperature and salinity on survival has been seen for other parasites (Möller 1978; Soleng and Bakke 1997). Our experimental setup did not allow this interaction, if real, to be tested. The interval between 4 °C, at which temperature the *P. perurans* population is sustained, and 8 °C, where positive amoebae population growth occurs should be investigated in more detail, as this temperature range may be important in better understanding potential reservoirs or overwintering risks during winter and spring months, in relation to temperature and freshwater inflow fluctuations.

Amoebae were observed after 24 h at a salinity of 3‰ at all temperatures investigated. An exposure time of 24 h is considerably longer than freshwater treatment times of 2 to 4 h (Oldham et al. 2016) used for AGD in the field, and the survival of some amoebae at 3‰ even after 24 h supports the use of salinities of less than 3‰, moreover as host gill mucus may provide added protection (Parsons et al. 2001; Best 2002 in Powell et al. 2005). However, laboratory and field studies have shown that local water chemistry can increase *Paramoeba* survival at lower salinities and even at 0‰ for 2.5–3 h, in the presence of elevated Ca^{2+} or Mg^{2+} ions (hardwater) (Green et al. 2005; Powell and Clark 2003; Roberts and Powell 2003). Reduction in Ca_2^+ concentrations, even when osmolarity is maintained, has been shown to reduce attachment and movement in other amoebae species e.g. *Vannella simplex* (Oshima et al. 1986). Salinities below 20–25‰ represent a stressor to *P. perurans*, based on the current work, and therefore may represent relevant salinities to be used in combination with other treatments such as H_2O_2, to bring about control.

The current experiments also do not inform on the ability of amoebae to recover growth, and infectivity following exposure to lower salinities/temperatures. Buchwald et al. (2015) observed an inverse relationship between growth recovery and suboptimal temperature exposure for *P. invadens*. Lima et al. (2016) reported recovery of typical amoeba morphology and attachment in *P. perurans* exposed to freshwater in vitro, with % recovery negatively correlated with exposure time, from 87% recovery after 0.5hr to no recovery after 4 h. High percentages of *P. perurans* survived over the 15 day experimental period at salinities of 20‰ or higher (dependent on temperature), therefore transfer of infected salmon to brackish waters as a control method may require that the water is less than 20‰ to prevent *P. perurans* population growth, and duration of placement in the water may need to extend well beyond 15 days. *Paramoeba perurans* and *P. pemaquidensis* have been observed forming rounded (temporary) cyst-like structures when exposed to freshwater or low salinities (Kent et al. 1988; Lima et al. 2016), and rounded amoebae forms have also been observed by Oshima et al. (1986) and Hauer and Rogerson (2005) in non-optimal osmotic concentrations, which were observed to regain normal trophozoite morphology and attachment/movement on return to suitable conditions. Rounded detached *P. perurans*, still viable based on neutral red staining, were observed at salinities below 20–25‰, and at low temperatures of 2–4 °C in this study. Therefore, while the outcome of non-optimal salinities may be a reduction in growth of the amoebae population, and eventual decline, this may be due both to physiological changes detrimental to viability itself, but also due to inability of amoebae to attach to substrate in their rounded state, to feed and divide (Dyková et al. 2000; Pickup et al. 2007; Rogerson et al. 2003). As suggested by some authors, the rounding and detachment of amoebae in detrimental environments may be a way of escaping conditions through dispersal away from the hostile environment.

As with most laboratory based experiments there are caveats in transferring findings to the field. These can include adaptation or selection of amoebae to long term stable culture conditions, which may obscure the capacity of field populations to survive and grow within extended ranges of environmental conditions (Hauer and Rogerson 2005). Amoebae growth can be modified by the presence and concentration of their prey species (Jellett and Scheibling 1988; Paniagua et al. 2001; Pickup et al. 2007), both of which can be influenced by the initial axenic isolation conditions, culturing conditions and changes in temperature and salinity. The presence of a host environment may promote higher growth rates as suggested with *P. pemaquidensis* fed on gill mucus or rainbow trout cell line (Lee et al. 2006, 2008). The gill microenvironment may increase protection from local short-term changes in salinity and detachment, by the presence of divalent cations in mucus, increasing salinity thresholds for survival/attachment at low salinities (Best 2002 in Powell et al. 2005). Previously it had been suggested that Intralamellar Vesicles (ILVs), formed by fusion of the gill lamellae may have protected the amoebae (Parsons et al. 2001), however these ILVs were subsequently shown to fully enclose the amoebae within an epithelial layer, with evidence of infiltration of immune cells (Adams and Nowak 2001), indicating that amoebae do not escape from these structures.

At growth permissive salinities and temperatures, amoebae numbers stabilised over the course of this experiment, rather than continuing to increase exponentially. This may be due to the closed nature of the in vitro system and build-up of toxins and metabolites from amoebae and bacteria, contact inhibition between amoebae signalling detachment, and or depletion of preferred prey items. Therefore, growth rate in the exponential stage, rather than final numbers is most important in identifying conditions suitable for population growth.

An observation from the data obtained was that least variation in amoebae population growth between replicate flasks 15 days post seeding was found at 15 °C. It could be hypothesised that this may represent homogenisation of the replication response in all amoebae, the culture having been maintained at this temperature for some time prior to the current experiment. When grown at other temperatures, the variation seen may reflect different degrees of plasticity within subpopulations to adapt and grow. However, there are insufficient data to offer any rigorous support for this interpretation.

Despite the issues highlighted above, the results here should provide a good indication of the salinity and temperature ranges for *P. perurans* population survival and growth. Additional studies would help better define these ranges in different geographical and environmental settings, and would help in understanding the ability of *P. perurans* to acclimatise or adapt to local or changing conditions, with associated changes in AGD risk and infection dynamics.

Methods

Nomenclature and definitions: There is an ongoing discussion on the taxonomy of paramoebid species. Species in the originally described genus of *Paramoeba* were subsequently divided into the two genera *Paramoeba* and *Neoparamoeba* based on the presence of surface microscales or of a glycocalyx respectively (Page 1983, 1987). However, an examination of published gene trees based on ribosomal DNA (Feehan et al. 2013; Volkova and Kudryavtsev 2017) does not indicate strong support for this division. While Feehan et al. (2013) suggests to lump together *Paramoeba* and *Neoparamoeba*, retaining the original *Paramoeba* nomenclature, Young et al. (2014) and Volkova and Kudryavtsev (2017) suggest that this would be premature due to insufficient sampling of scaled amoeba species which may improve phylogenetic resolution of the genera. For the purpose of this manuscript we adopt the nomenclature of Feehan et al. (2013), and use the genus *Paramoeba* throughout. This is based on it being the simplest taxonomy, where evidence is limited to support either of the above arguments.

The terms “sustained”, “survival” and “growth” used hereafter refer to the numbers of *P. perurans*, rather than individual amoebae. For example “growth” indicates an increase in the number of amoebae over time rather than increases in amoeba cell volume or other parameters indicative of increased cellular material in individual amoebae, and “sustained” to a population maintaining amoebae numbers (not increasing or decreasing) rather than the long-term survival of individual amoebae. The term “viable” refers to uptake of vital stains. It is assumed that the amoebae are alive but does not guarantee that viable amoebae will function normally in relation to biological or infective parameters.

Paramoeba perurans: An in vitro culture system was used as a proxy for *Paramoeba perurans* population growth in the field under different temperature and salinity conditions.

A *P. perurans* culture was established at Marine Scotland Science in November 2012 from gills of infested farmed Atlantic salmon from the west coast of Scotland. Cultures were isolated as described in Crosbie et al. (2012) and Collins et al. (2017) and maintained on malt yeast agar (MYA) underlay at 15 °C in full sea water. The culture was not axenic and contained contaminating microflora, co-isolated with amoebae from fish gills. All observed amoebae were paramoebid-like in appearance. Confirmation of *P. perurans* in culture was performed by 18S rDNA species specific QPCR (Fringuelli et al. 2012), and traditional PCR (Young et al. 2008a). PCR analysis was performed on culture samples from a range of salinity and temperature combinations, taken at the end of the growth and survival experiments. All produced strong amplification products for *P. perurans.* In addition, PCR amplification was negative for *Paramoeba pemaquidensis* (Wong et al. 2004) and *Paramoeba branchiphila* (Dyková et al. 2005), *Paramoeba atlantica* and *Paramoeba eilhardi*. PCR assays were designed in-house for *P. atlantica* (P. atl F1: 5′- GGTGATAATTTGGTTTTCTTTCG-3′, P. atl R1: 5′- ACCGAAATCAATAAGTAACTGTCTC-3′) and *P. eilhardi* (P. eil F1: 5′- GAGTCGTCTCTTCGGAGGAGT-3′, P. eil R1: 5′- CGAAATCGCAAGTYATTCTGACG-3′) using cultures CCAP 1560/9 and CCAP 1560/2 respectively, obtained from the Culture Collection of Algae and Protozoa (CCAP: https://www.ccap.ac.uk/), as positive controls.

Experimental outline: temperature and salinity effect on *P. perurans* population growth: The set of experiments to examine the effect of temperature and salinity on the population growth of *P. perurans* was initiated in February 2014, approximately 14 months after establishment of culture. All combinations of the following temperatures (T) and salinities (S) were tested: T (2, 4, 8, 11, 15, 18 °C) and S (0, 3, 5, 15, 20, 25, 30, 35, 40, 45, 50‰).

Each combination of temperature and salinity was tested in triplicate. Due to the extended duration of the experiment (i.e. exposure of amoebae to different temperature and salinity combinations was performed in blocks over time to allow for constraints on incubator and staff availability), the temperatures (2–3 temperatures per block) and salinities were randomised over blocks (Fig. S1—supplementary material). This was to mitigate for artifactual trends due to any potential shift in the amoebae culture properties over the time course of the experiment. Statistically, the experiment can be regarded as an unbalanced incomplete block design.

Exposure of *P. perurans* to different temperatures and salinities: Amoebae cultures were acclimatised, to temperature only, for 2 days before experiment start. On day 0 of the experimental period, amoebae attached to MYA were detached by tapping the flasks repeatedly against a hard surface and approximately 3000 amoebae mL^{-1} consisting of the resuspended amoebae and those already in suspension, were seeded in 25 cm^2 tissue culture flasks (Greiner) on a MYA underlay made with filtered seawater of required salinity, and with an overlay of 7 mL of filtered seawater adjusted to required salinity, with deionised water (0‰ = 100% deionised water), or with addition of artificial sea salts (Sigma) for salinities greater than 35‰. The flasks were placed in incubators set at required temperatures. Amoebae were observed over a 15 day period with counts taken at days 1–9, 12 and 15.

Amoebae counts: At each timepoint, each flask was inverted briefly (i.e. to transfer water overlay from the MYA surface to opposite surface) and shaken gently to mix cells in suspension while minimising dislodgement of attached amoebae on agar surface. The flask was placed upright and 100 μL of sea water overlay (1.43% of the total overlay volume) containing amoebae was removed for counting. The aliquot was added to 0.35 μL of the vital stain neutral red (NR) (Sigma–Aldrich, Germany) and placed in the dark for 30 min to allow NR uptake by living cells. This was followed by centrifugation of the stained amoebae at 400 g for 10 min, removal of the supernatant and resuspension of amoebae in 100 μL of 0.22 μm filtered seawater, adjusted to appropriate salinity. The resuspended stained amoebae were transferred to a 96-well plate, and 10 fold serially diluted (into filtered seawater of the same salinity (0‰ = 100% deionised water)) to 10^{-2}. The amoebae were allowed to settle for 30 min before counting. Viable amoebae were distinguished by uptake of NR stain. Total settled amoebae (divided into live and dead based on staining) were counted in each well, or, where numbers were too high to count, were counted in the 10^{-1} and 10^{-2} dilutions only. The amoebae numbers in dilutions were adjusted to represent amoebae numbers in 100 μL (1.43%) of original suspension.

Photos of attached amoebae were taken at approximately 2 h post seeding and then daily from day 1–9, and on days 12 and 15. Ten fields of view were photographed, at 10× magnification, at regular (marked with pen) intervals across a diagonal transect of the flask, as amoebae had been observed to be unevenly distributed in flasks. All amoebae in each field of view were counted, and it was assumed that all attached amoebae were alive. This represented in total 3.25 mm^2 (1.30%) of the underlay surface area.

Every two days 200 uL of heat inactivated bacteria (*Flavobacterium psychrophilum*), suspended in appropriate salinity sea water at a concentration of 10^6 CFU mL^{-1}, were added to cultures as a food source and to maintain volume of culture sea water overlay following removal of aliquots for amoebae counting (see below).

Analysis: Data. Observations for analysis comprised counts of the number of putative viable *P. perurans* incubated at different combinations of temperatures and salinities, listed previously, at the 12 post-inoculation time-points. To analyse *P. perurans* population survival/growth, standardised underlay and live overlay counts were summed, generating a number of total live amoebae for a 1.43% sample of each flask, apart from one analysis where survival/growth over time was presented for attached (1.43%) and suspended (1.43%) amoebae separately. This was then transformed, for statistical analysis, by the addition of 1.01 and conversion to the $\log_2$ scale. Data for the day of inoculation (day 0) were omitted from analyses because counts for the overlay were missing. Occasional missing values were encountered due to inadvertent errors in counting process, and are apparent from the result plots. **Statistical analyses** were performed within the R Statistical Environment (Ihaka and Gentleman 1996), version 3.2.3 (R Core Team 2015), utilising the supplementary R package mgcv version 1.8-9 (Wood 2015) as required.

Initial attempts to analyse the complete data using generalized linear mixed-effect models, simultaneously incorporating the many sources of biological and experimental variation were unsuccessful. The analysis was therefore broken down into several individual components using simpler models in which experimental variation between replicate flasks, arising from random between-block and between-flask-within-block effects, was treated as a single 'nuisance' error. Four analyses were undertaken. **Persistence of *P. perurans* population at different temperature and salinity combinations at one day post exposure.** Changes in transformed total amoeba number on day one with increases in salinity for individual temperatures were modelled using a three-parameter logistic equation estimated by non-linear least squares. The rationale for this, supported from preliminary plots of day one data, is that survival is very low at salinities of 0‰ and plateaus at higher values. The 95% confidence intervals (CI) of predicted transformed amoeba number were estimated using a non-parametric bootstrap (Efron 1979). Estimates were converted back to predicted total amoeba number and plotted on the $\log_2$ scale. **Persistence of *P. perurans* population at different temperature and salinity combinations at 15 days post exposure.** Changes in transformed total amoeba number on day 15 were analysed as described above. Additionally, approximate minimum salinities permissive of sustaining *P. perurans* populations were estimated. The rationale underlying the calculations is that amoebae numbers at day 15 will be no fewer than those at day one at salinities capable of maintaining population numbers. The minimum permissive salinity was therefore calculated as the salinity, greater than 15‰, where the predicted values from the fitted model for day 15 were most similar to those for the model for day 1. The 95% CI were estimated by bootstrapping both models. Salinities greater than 15‰ only were considered because additional intersects arising from the variability of low counts may occur at lower salinities (e.g. 0‰). **Persistence of *P. perurans* population at different temperature and salinity combinations over time.** The pattern of change in transformed attached and suspended amoeba number over time for individual temperatures and salinities was characterised. The functional forms of the change over time, while assumed to be relatively simple, are not known and a non-parametric approach to modelling these data was therefore adopted. Transformed attached and viable suspended amoeba numbers over the 15 day incubation period at individual temperatures and salinities were modelled as penalised cubic regression splines with a maximum of three degrees of freedom within a generalized additive model framework (Wood 2011) (GAM) assuming a normal error distribution by maximum likelihood. The 95% CI of predicted transformed amoeba numbers are available from the estimates of standard error around splines. Estimates were converted to predicted attached and suspended amoeba numbers and plotted on the $\log_2$ scale. **Minimum doubling times of *P. perurans* population under different temperature and salinity combinations.** Minimum doubling times of transformed total amoeba number for individual salinity and temperature combinations were estimated. The rationale for the calculations is that minimum doubling times will correspond to maximum growth rates during the 15 day culture period and that a comparison of these provides information on optimal temperature and salinity combinations for population growth. Transformed total amoeba numbers over time at individual salinity and temperature combinations were modelled using a four parameter logistic equation. The equation differs from the three parameter equation used previously by incorporating a lower non-zero positive value which facilitates the modelling of amoeba numbers, which may be present in high numbers on day one, over time. The equation was fitted to multiple (1000 per salinity and temperature combination) bootstrapped data sets with sampling taking place within each day. Equation parameters from each bootstrap replication were then used to determine the gradients at the inflection of the curves using a numerical approximation of the first derivative which were then converted into a doubling times in hours. The median doubling time for a temperature-salinity combination with 95% CI based on the 2.5% and 97.5% quantiles was then calculated.

Results are only provided for temperature-salinity combinations at which amoeba numbers increased over time and for which a majority (>50%) of bootstrap samples generated an estimate. Additionally estimates of minimum doubling times at 4 °C (for 40‰) and 18 °C (for 45‰) are excluded because the 95% CI were sufficiently large as to render the estimates uninformative and no other estimates at the same temperature were available. In practice this limits doubling time estimates to temperatures of 8 to 15 °C and salinities of 25 to 50‰ inclusive.

Declarations of Interest

None.

Acknowledgements

This work was funded under Scottish Government grant AQ0080, and by Marine Harvest Ltd. The authors also acknowledge Marine Harvest Ltd. for provision of infected material from which *Paramoeba perurans* cultures were isolated. The ability of the bacterial food source, *Flavobacterium psychrophilum*, to facilitate amoebae growth was established in collaboration with an MSc student (Valentina Romano) at the Institute of Aquaculture, University of Stirling, UK.

Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.protis.2018.11.003.

References

Adams MB, Nowak BF (2001) Distribution and structure of lesions in the gills of Atlantic salmon, *Salmo salar* L. , affected with amoebic gill disease. J Fish Dis **24**:535–542

Adams MB, Nowak BF (2003) Amoebic gill disease: sequential pathology in cultured Atlantic salmon, *Salmo salar* L. J Fish Dis **26**:601–614

Best G (2002) Effects of Water Chemistry and Salmon Mucus on the Survival of a Cultured Strain of *Neoparamoeba pemaquidensis* (PA027). M. Appl. Sci. Thesis. University of Tasmania

Bridle A, Crosbie P, Cadoret K, Nowak B (2010) Rapid detection and quantification of *Neoparamoeba perurans* in the marine environment. Aquaculture **309**:56–61

Buchwald RT, Feehan CJ, Scheibling RE, Simpson AGB (2015) Low temperature tolerance of a sea urchin pathogen: Implications for benthic community dynamics in a warming ocean. J Exp Mar Biol Ecol **469**:1–9

Bustos PA, Young ND, Rozas MA, Bohle HM, Ildefonso RS, Morrison RN, Nowak BF (2011) Amoebic gill disease (AGD) in Atlantic salmon (*Salmo salar*) farmed in Chile. Aquaculture **310**:281–288

Cann JP, Page FC (1982) Fine structure of small free-living *Paramoeba* (Amoebida) and taxonomy of the genus. J Mar Biol Assoc UK **62**:25–43

Clark A, Nowak BF (1999) Field investigations of amoebic gill disease in Atlantic salmon, *Salmo salar* L. , in Tasmania. J Fish Dis **22**:433–443

Clark G, Powell M, Nowak B (2003) Effects of commercial freshwater bathing on reinfection of Atlantic salmon, *Salmo salar*, with amoebic gill disease. Aquaculture **219**:135–142

Collins C, Hall M, Bruno D, Sokolowska J, Duncan L, Yuecel R, McCarthy U, Fordyce MJ, Pert CC, McIntosh R, MacKay Z (2017) Generation of *Paramoeba perurans* clonal cultures using flow cytometry and confirmation of virulence. J Fish Dis **40**:351–365

Crosbie PBB, Ogawa K, Nakano D, Nowak BF (2010) Amoebic gill disease in hatchery-reared ayu, *Plecoglossus altivelis* (Temminck & Schlegel), in Japan is caused by *Neoparamoeba perurans*. J Fish Dis **33**:455–458

Crosbie PBB, Bridle AR, Cadoret K, Nowak BF (2012) In vitro cultured *Neoparamoeba perurans* causes amoebic gill disease in Atlantic salmon and fulfils Koch's postulates. Int J Parasitol **42**:511–515

Crosbie PBB, Bridle AR, Nowak BF (2014) Atlantic Salmon Aquaculture Subprogram: Culture and cryopreservation of *Neoparamoeba perurans* (AGD). Final report FRDC PROJECT NUMBER:2012/048: http://www.frdc.com.au/project?id=561, Accessed 16 February 2018.

Douglas-Helders M, Nowak B, Butler R (2005) The effect of environmental factors on the distribution of *Neoparamoeba pemaquidensis* in Tasmania. J Fish Dis **28(10)**:583–592

Douglas-Helders M, Saksida S, Raverty S, Nowak BF (2001) Temperature as a risk factor for outbreaks of amoebic gill disease in farmed Atlantic salmon (*Salmo salar*). Bull Eur Assoc Fish Pathol **21**:114–116

Douglas-Helders GM, Dawson DR, Carson J, Nowak BF (2002) Wild fish are not a significant reservoir for *Neoparamoeba pemaquidensis* (Page, 1987). J Fish Dis **25**: 569–574

Douglas-Helders GM, O'Brien DP, McCorkell BE, Zilberg D, Gross A, Carson J, Nowak BF (2003) Temporal and spatial distribution of paramoebae in the water column—a pilot study. J Fish Dis **26**:231–240

Downes JK, Rigby ML, Taylor RS, Maynard BT, MacCarthy E, O'Connor I, Marcos-Lopez M, Rodger HD, Collins E, Ruane NM, Cook MT (2017) Evaluation of non-destructive molecular diagnostics for the detection of *Neoparamoeba perurans*. Front Mar Sci **4**:1–9

Dyková I, Figueras A, Peric Z (2000) *Neoparamoeba* Page, 1987: light and electron microscopic observations on six strains of different origin. Dis Aquat Org **43**:217–223

Dyková I, Nowak BF, Crosbie PBB, Fiala I, Peckov H, Adams MB, Macháčková Dvořáková H (2005) *Neoparamoeba branchiphila* n. sp., and related species of the genus *Neoparamoeba* Page, 1987: morphological and molecular characterization of selected strains. J Fish Dis **28**:49–64

Efron B (1979) Bootstrap methods: another look at the jacknife. Ann Stat **7**:1–26

Feehan CJ, Scheibling RE, Brown MS, Thompson KR (2016) Marine epizootics linked to storms: mechanisms of pathogen introduction and persistence inferred from coupled physical and biological time-series. Limnol Oceanogr **61**:316–329

Feehan CJ, Johnson-Mackinnon J, Scheibling RE, Lauzon-Guay JS, Simpson AG (2013) Validating the identity of Paramoeba invadens, the causative agent of recurrent mass mortality of sea urchins in Nova Scotia, Canada. Dis Aquat Org **103**:209–227

Fringuelli E, Gordon AW, Rodger H, Welsh MD, Graham DA (2012) Detection of *Neoparamoeba perurans* by duplex quantitative Taqman real-time PCR in formalin-fixed, paraffin-embedded Atlantic salmonid gill tissues. J Fish Dis **35**: 711–724

Green TJ, Powell MD, Harris JO, Butler R (2005) Effects of dissolved organic carbon and hardness in freshwater used to treat amoebic gill disease. Aquat Res **36**:398–404

Haugland GT, Olsen AB, Rønneseth A, Andersen L (2017) Lumpfish (*Cyclopterus lumpus* L.) develop amoebic gill disease (AGD) after experimental challenge with *Paramoeba perurans* and can transfer amoebae to Atlantic salmon (*Salmo salar* L.). Aquaculture **478**:48–55

Hauer G, Rogerson A (2005) Remarkable salinity tolerance of seven species of naked amoebae (gymnamoebae). Hydrobiologia **549**:33–42

Hellebø A, Stene A, Aspehaug V (2017) PCR survey for *Paramoeba perurans* in fauna, environmental samples and fish associated with marine farming sites for Atlantic salmon (*Salmo salar* L.). J Fish Dis **40**:661–667

Ihaka R, Gentleman R (1996) R: A language for data analysis and graphics. J Comput Graph Stat **5**:299–314

International Council for the Exploration of the Sea (ICES) (2015) Report of the Working Group on Pathology and Diseases of Marine Organisms (WGPDMO), Helsinki, Finland. ICES CM 2015/SSGEPI:01. 124 p.

Jellett JF, Scheibling RE (1988) Effect of temperature and prey availability on growth of *Paramoeba invadens* in monoxenic culture. Appl Environ Microbiol **54**:1848–1854

Karlsbakk E, Olsen AB, Einen ACB, Mo TA, Fiksdal IU, Aase H, Kalgraff C, Skår SA, Hansen H (2013) Amoebic gill disease due to *Paramoeba perurans* in ballan wrasse (*Labrus bergylta*). Aquaculture **412–413**:41–44

Kent ML, Sawyer TK, Hedrick RP (1988) *Paramoeba pemaquidensis* (Sarcomastigophora: Paramoebidae) infestation of the gills of coho salmon *Oncorhynchus kisutch* reared in sea water. Dis Aquat Organ **5**:163–169

Kim WS, Kong KH, Kim JO, Jung SJ, Kim JH, Oh MJ (2017) Amoebic gill disease outbreak in marine fish cultured in Korea. J Vet Diag Invest **29**:357–361

Lee LEJ, Van Es SJ, Walsh SK, Rainnie DJ, Donay N, Summerfield R, Cawthorn RJ (2006) High yield and rapid growth of *Neoparamoeba pemaquidensis* in co-culture with a rainbow trout gill-derived cell line RTgill-W1. J Fish Dis **29**: 467–480

Lee LEG, Bufalino MR, Inthavong B, Jones N, Nathanson D (2008) Growth of *Neoparamoeba* spp. for paramoebiasis research with fish and shellfish cell cultures. The Bulletin, MDI Biological Laboratory **47**:2008

Lima PC, Taylor RS, Cook M (2016) Involvement of contractile vacuoles in the osmoregulation process of the marine parasitic amoeba *Neoparamoeba perurans*. J Fish Dis **39**:629–633

Martin RE (1985) Population growth in stationary and suspension culture of *Paramoeba pemaquidensis* Page (Amoebida, Paramoebidae). J Protozool **32**:738–739

Möller H (1978) The effects of salinity and temperature on the development and survival of fish parasites. J Fish Biol **12**:311–323

Mouton A, Crosbie P, Cadoret K, Nowak B (2013) First record of amoebic gill disease caused by *Neoparamoeba perurans* in South Africa. J Fish Dis **37**:407–409

Mullen TE, Nevis KR, O'Kelly CJ, Gast RJ, Frasca S Jr (2005) Nuclear small-subumit ribosomal RNA gene-based characterisation, molecular phylogeny and PCR detection of the *Neoparamoeba* from western Long Island Sound lobster. J Shellfish Res **24**:719–731

Munday BL, Zilberg D, Findlay V (2001) Gill disease of marine fish caused by infection with *Neoparamoeba pemaquidensis*. J Fish Dis **24**:497–507

Nowak B, Bryan J, Jones S (2010) Do salmon lice, *Lepeophtheirus salmonis*, have a role in the epidemiology of amoebic gill disease caused by *Neoparamoeba perurans*? J Fish Dis **33**:683–687

Nowak BF (2012) *Neoparamoeba perurans*. In Woo PTK, Buchmann K (eds) Fish Parasites: Pathobiology and Protection. CAB International, Wallingford, pp 1–18

Oldham T, Rodger H, Nowak BF (2016) Incidence and distribution of amoebic gill disease (AGD)—An epidemiological review. Aquaculture **457**:35–42

Oshima N, Takeda F, Ishii K (1986) Responses of freshwater amoebae to salinity changes. Comp Biochem Physiol **85A**:395–399

Page FC (1983) Marine Gymnamoebae. Institute of Terrestrial Ecology, Cambridge ISBN 0 904282 759.

Page FC (1987) The classification of the 'naked' amoeba of the phylum Rhizapoda. Arch Protistenkd **133**:199–217

Paniagua E, Parama A, Iglesias R, Sanmartin ML, Leiro J (2001) Effects of bacteria on the growth of an amoeba infecting the gills of turbot. Dis Aquat Org **45**:73–76

Parsons H, Nowak B, Fisk D, Powell M (2001) Effectiveness of commercial freshwater bathing as a treatment against amoebic gill disease in Atlantic salmon. Aquaculture **195**: 205–210

Pert C, Allcock Z, Schrittwieser M, McCarthy U, Collins C (2015) Assessment of the viability of the different life stages of *Lepeophtheirus salmonis* following exposure to hydrogen peroxide. A study commissioned by the Scottish Aquaculture Research Forum (SARF) http://www.SARF.org/.

Pickup ZL, Pickup R, Parry JD (2007) A comparison of the growth and starvation responses of *Acanthamoeba castellanii* and *Hartmannella vermiformis* in the presence of suspended and attached *Escherichia coli* K12. FEMS Microbiol Ecol **59**:556–563

Powell MD, Clark GA (2003) In vitro survival and the effect of water chemistry and oxidative chemical treatments on isolated gill amoebae from AGD-affected Atlantic salmon. Aquaculture **220**:135–144

Powell MD, Attard M, Harris JO, Roberts SD, Leef MJ (2005) Why fish die – the treatment and pathophysiology of AGD. Aquafin CRC Project 3.4.1 (FRDC project No. 2001/205): ISBN 1 86295 259 0.

R Core Team (2015) A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna. https://www.R-project.org/.

Roberts S, Powell M (2003) Reduced total hardness of fresh water enhances the efficacy of bathing as a treatment for amoebic gill disease in Atlantic salmon, *Salmo salar* L. J Fish Dis **26**:591–599

Rodger HD (2014) Amoebic gill disease (AGD) in farmed salmon (*Salmo salar*) in Europe. Fish Vet J **14**:16–27

Rodger HD, McArdle J (1996) An outbreak of amoebic gill disease in Ireland. Vet Rec **139**:348–349

Rogerson A, Anderson OR, Vogel C (2003) Are planktonic naked amoebae predominately floc associated or free in the water column? J Plankton Res **25**:1359–1365

Sætre R, Ljøen R (1972) The Norwegian Coastal Current. Proceedings from the First International Conference on Port and Ocean Engineering under Arctic Conditions **1**: 514–535

Sætre R, Aure J, Danielssen DS (2003) Long-term hydrographic variability patterns off the Norwegian coast and in the Skagerrak. ICES Marine Science Symposia **219**: 150–159

Scheibling RE, Lauzon-Guay J-S (2010) Killer storms: North Atlantic hurricanes and disease outbreaks in sea urchins. Limnol Oceanogr **55**:2331–2338

Shinn AP, Pratoomyot J, Bron JE, Paladini G, Brooker EE, Brooker AJ (2015) Economic costs of protistan and metazoan parasites to global mariculture. Parasitology **142**: 196–270

Soleng A, Bakke TA (1997) Salinity tolerance of *Gyrodactylus salaris* (Platyhelminthes, Monogenea): laboratory studies. Can J Fish Aquat Sci **54**:1837–1845

Stagg HEB, Hall M, Wallace IS, Pert CC, Garcia Perez S, Collins C (2015) Detection of *Paramoeba perurans* in Scottish marine wild fish populations. Bull Eur Assoc Fish Pathol **35**:217–226

Steinum T, Kvellestad A, Rønneberg LB, Nilsen H, Asheim A, Fjell K, Nygård SMR, Olsen AB, Dale OB (2008) First cases of amoebic gill disease (AGD) in Norwegian seawater farmed Atlantic salmon, *Salmo salar* L. , and phylogeny of the causative amoeba using 18S cDNA sequences. J Fish Dis **31**: 205–214

Tan CK, Nowak BF, Hodson SL (2002) Biofouling as a reservoir of *Neoparamoeba pemaquidensis* (page, 1970), the causative agent of amoebic gill disease in Atlantic salmon. Aquaculture **210**:49–58

Wong FYK, Carson J, Elliott NG (2004) 18S ribosomal DNA-based PCR identification of *Neoparamoeba pemaquidensis*, the agent of amoebic gill disease in sea-farmed salmonids. Dis Aquat Org **60**:65–76

Young ND, Crosbie PBB, Adams MB, Nowak BF, Morrison RN (2007) *Neoparamoeba perurans* n. sp., an agent of amoebic gill disease of Atlantic salmon (*Salmo salar*). Int J Parasitol **37**:1469–1481

Young ND, Dykova I, Nowak BF, Morrison RN (2008a) Development of a diagnostic PCR to detect *Neoparamoeba perurans*, agent of amoebic gill disease. J Fish Dis **31**: 285–295

Young ND, Dyková I, Snekvik K, Nowak BF, Morrison RN (2008b) *Neoparamoeba perurans* is a cosmopolitan aetiological agent of amoebic gill disease. Dis Aquat Org **78**: 217–223

Young ND, Dyková I, Crosbie PB, Wolf M, Morrison RN, Bridle AR, Nowak BF (2014) Support for the coevolution of *Neoparamoeba* and their endosymbionts, Perkinsela amoebae-like organisms. Europ J Protistol **50**: 509–523

VKM (2014) Panel on Animal Health and Welfare; Risk assessment of amoebic gill disease, VKM Report 2014: 11 [39 pp], ISBN nr. 978-82-8259-149-2, Oslo, Norway. Available online: www.vkm.no.

Volkova E, Kudryavtsev A (2017) Description of *Neoparamoeba longipodia* n. sp. and a new strain of *Neoparamoeba aestuarina* (Page, 1970) (Amoebozoa, Dactylopodida) from deep-sea habitats. Europ J Protistol **61**:107–121

Wood SN (2011) Fast stable restricted maximum likelihood and marginal likelihood estimation of semiparametric generalized linear models. J R Stat Soc Ser B **73**:3–36

Wood SN (2015) mgcv: Mixed GAM Computation Vehicle with GCV/AIC/REML Smoothness Estimation. https://CRAN.R-project.org/package=mgcv.

Wright D, Nowak B, Oppedal F, Bridle A, Dempster T (2015) Depth distribution of the amoebic gill disease agent, *Neoparamoeba perurans*, in salmon sea cages. Aquat Environ Interact **7**:67–74