Atlantic salmon (*Salmo salar* L.) exposed to cultured gill-derived *Neoparamoeba branchiphila* fail to develop amoebic gill disease (AGD)

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**Abstract**

Gill-derived *Neoparamoeba* spp. from Atlantic salmon cause amoebic gill disease (AGD) in naïve recipients. Atlantic salmon were inoculated with clonal gill-derived *Neoparamoeba branchiphila* that had been cultured in the presence or absence of Atlantic salmon cutaneous mucus. *Neoparamoeba branchiphila* did not elicit AGD and the supplementation of cultures with cutaneous mucus did not influence virulence.

Amoebic gill disease (AGD) of Atlantic salmon is caused by amphizoic marine amoebae, *Neoparamoeba* spp. (Adams & Nowak, 2004; Dyková, et al., 2005). In Tasmania, sea-cage cultured Atlantic salmon are predominantly affected by AGD during summer months in association with a rise in water temperature and salinity to 35 ‰ (Clark & Nowak, 1999; Adams & Nowak, 2003). Freshwater bathing is the only treatment currently available for alleviating AGD and the development of an AGD vaccine would provide substantial benefits for the Tasmanian salmon growers. There is evidence that Atlantic salmon can develop an adaptive immune response to gill-derived (wild-type) *Neoparamoeba* spp. Some Atlantic salmon develop a serum antibody response after experimental inoculation or natural exposure to wild-type *Neoparamoeba* spp. in the culture environment, however the response is slow to develop and serum antibody levels are low (Vincent et al., 2006; Vincent et al., 2007). Further, intra-peritoneal (i.p) immunisation of Atlantic salmon with a crude wild-type preparation in the presence of adjuvant stimulates a significant serum antibody response (Akhlaghli, et al., 1996). Currently, the only source of virulent *Neoparamoeba* spp. is gill-derived amoebae that are obtained from AGD-affected Atlantic salmon (Morrison, et al., 2004) and assessment of amoebae preparations that stimulate a significant immune response is restricted by the availability of infectious parasites. Maintaining a virulent strain of *Neoparamoeba* spp. in culture would provide access to high cell densities and eliminate the need for Atlantic salmon as hosts for the passage of wild-type parasites. Both *Neoparamoeba branchiphila* and *N. pemaquidensis* have been cultured from the gills of AGD-affected Atlantic salmon (Dyková, et al., 2005) and therefore AGD may be a condition of mixed

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etiology. Cultured *N. pemaquidensis* tested to date fail to elicit AGD in Atlantic salmon (Kent, et al., 1988; Findlay, 2001; Howard et al., 1993; Morrison, et al., 2005) however, the virulence of *N. branchiphila* has not yet been assessed. This work was conducted to firstly identify if AGD could be elicited in Atlantic salmon by inoculation with a culture strain of *N. branchiphila* (NRSS/II) (Dyková, et al., 2005) and secondly to determine if supplementing the cultures with cutaneous mucus from Atlantic salmon influences virulence.

At present, a tank of Atlantic salmon at the University of Tasmania (Launceston, Australia) is used as a source of wild-type *Neoparamoeba* spp. (UTAS cohabitation tank). Naïve fish placed in this tank are infected during cohabitation and wild-type *Neoparamoeba* spp. are routinely isolated by plastic adherence (Morrison et al., 2004). Partially purified trophozoites supply wild-type *Neoparamoeba* spp. for infection trials and *in vitro* investigations. *Neoparamoeba branchiphila* (NRSS/II) is a cultured clonal strain that originated from the gills of Atlantic salmon housed in the UTAS cohabitation tank. Amoebae were maintained on sea water malt yeast agar; 75% (v/v) coarse-filtered sea water (35‰), 25% (v/v) distilled water, 0.01% (w/v) malt, 0.01% (w/v) yeast (Oxoid, Hampshire, England), 2% (w/v) Bacto agar (Becton, Dickson & Co., Sparks, Maryland, USA). *Neoparamoeba branchiphila* were harvested by washing the agar with sterile seawater using a transfer pipette, concentrated by centrifugation at 500’ g for 5 min and enumerated by haemocytometer. Cells were placed in 50 mL tissue culture flasks containing 10 mL of sterile sea water including an antibiotic mix to reduce bacterial growth (Morrison, et al., 2005). As a control, an equal cell-density of *N. branchiphila* was cultured in the absence of antibiotics and mucus. Preliminary investigations identified that a >4-fold difference in growth was seen when cells were cultured in the presence of cutaneous mucus (data not shown). While bacteria support growth of cultured *Neoparamoeba* spp. (Kent et al., 1988; Morrison et al., 2005), we hypothesise that *in vivo*, *Neoparamoeba* spp. graze on gill mucus. Therefore, to provide culture conditions that may reflect the host environment, the culture media was then supplemented with 150 mL of autoclaved cutaneous mucus. Mucus was collected from anaesthetised (Aqui-S NZ Ltd, Lower Hutt, New Zealand) Atlantic salmon by gently scraping the skin with the edge of a glass slide. Anti-protease cocktail (Sigma-Aldrich) was added to the mucus suspension. *Neoparamoeba branchiphila* cultures were maintained at 18°C for 44 days and the culture media was replenished every 8-10 days.

Atlantic salmon (100-150g) were acclimatised to seawater at 35% salinity and 16°C in an independent recirculating system. For the infection trial, 3 systems, each comprising 3 ‘ 80L tanks connected to an 80L sump, were used. Two fish were placed in each of the tanks and each system was inoculated with 1) *N. branchiphila* that had been cultured with mucus (3000 cells/L), 2) *N. branchiphila* that had been cultured without mucus (3000 cells/L) and 3) no treatment. Water exchanges of approximately 25% of the system volume were conducted every second day and total ammonia concentrations over the trial period of 12 d were below 2 mg L⁻¹. Twelve days post-inoculation, fish were killed by overdose of
Aqui-S. The entire gill basket was fixed in seawater Davidson’s. Gills were then transferred to 70% ethanol after 24 h and processed following routine histological protocols. Sections (5 μm) were stained with H & E and assessed for the presence of *Neoparamoeba* spp. by light microscopy at 400x magnification.

Atlantic salmon exposed to a high cell density of *N. branchiphila* either cultured in the presence or absence of cutaneous mucus for 12 d did not develop gill pathology consistent with AGD. Fish that were not exposed to *N. branchiphila* also displayed normal gill structure. Failure to elicit AGD in the current study by inoculation with *N. branchiphila* was not due to the susceptibility of these fish, as representatives of this cohort were transferred to the UTAS cohabitation tank and became moribund with AGD in approximately 25 d. Atlantic salmon exposed to a similar cell density of wild-type *Neoparamoeba* spp. in the same experimental systems used here developed AGD within 8 d post-inoculation (Morrison et al., 2004, Morrison et al., 2005). Together this suggests that the cultured gill-derived *N. branchiphila* may be avirulent. In the current study, addition of crude cutaneous mucus in an attempt to mimic the host environment supported growth of *N. branchiphila* *in vitro* yet did not influence their capacity to cause AGD. Mechanisms of virulence and conditions that influence virulence of wild-type *Neoparamoeba* spp. are unknown. Wild-type *Neoparamoeba* spp. may graze on mucus, influencing virulence. However, heat-induced sterilisation of cutaneous mucus may have inhibited this (putative) effect.

This work formed part of a project of Aquafin CRC and was supported by the Australian Government’s CRC program, the Fisheries R & D Corporation and other CRC participants. The authors would like to thank M. Attard for technical support.

**References**


