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# Distribution of *Neoparamoeba* sp. in sediments around marine finfish farming sites in Tasmania

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ABSTRACT: Marine sediment samples collected from various sites at 2 Atlantic salmon farms in Tasmania were analysed for the presence of *Neoparamoeba* sp., an amoeba associated with amoebic gill disease (AGD) in farmed Atlantic salmon. Environmental variables of the sediment layer at each site, including redox potential and sulphide concentration, were measured and the general biological condition assessed by video observation. Sediments and environmental data were collected on 4 occasions at each site over a 12 mo period. *Neoparamoeba* sp. was detected in populations of amoebae recovered by culture from all sites and in 50% of all sediment samples taken. There was evidence of a seasonal influence on the presence of the amoeba, but this was different at each farm. No *Neoparamoeba* sp. was recovered from any sites at Farm 1 during the winter of 2002 whereas at Farm 2 this was the case for the summer of 2003. There appeared to be no relationship between the presence of *Neoparamoeba* sp., salmon farming activities and environmental parameters.

KEY WORDS: *Neoparamoeba* · Amoebic gill disease · AGD · Marine sediments · Environmental parameters



## INTRODUCTION

*Neoparamoeba* sp. is a marine gymnamoeba that is associated with amoebic gill disease (AGD) in farmed Atlantic salmon *Salmo salar* L. in south eastern Tasmania. For a number of years AGD has been the only major infectious disease affecting cultured salmon in Australia and it continues to have a severe impact (Munday et al. 1990, Munday et al. 2001, Nowak et al. 2002). The disease was first described in Tasmania by Munday (1986), and since then the incidence and prevalence of AGD has grown in parallel with salmon production. Interaction between the amoeba and the host can result in macroscopic multifocal white mucoid patches on the gills, leading to excess mucus production and respiratory distress (Munday et al. 1990). Freshwater bathing effectively controls the disease, but the increasing frequency of treatment is a financial burden for salmon growers. On farm leases, salmon are regularly inspected for AGD-characteristic white patches on gills and individual pens are rated or scored according to the severity or the quantity of white patches. Treatment can be triggered by high gill scores; often, however, regular bathing occurs routinely, regardless of severity of infection. In most cases, the freshwater is discharged within the lease on completion of treatment.

The presumptive causative agent of AGD is *Neoparamoeba* sp. Until recently, *Neoparamoeba pemaquidensis* was thought to be solely responsible, but a new species, also isolated from salmon gills with AGD, has recently been described and named *Neoparamoeba branchiphila* (Dyková et al. 2005). For the purpose of this paper both species are referred to solely as *Neoparamoeba* sp.. *Neoparamoeba* sp. is a common marine amoeba (Page 1983) and generally free-living, but can be parasitic in certain circumstances that are yet to be elucidated. In Tasmania *Neoparamoeba* sp. has been isolated and cultured from marine sediments from both farmed and non-farmed sites (Crosbie et al. 2003),

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from gills of AGD-affected Atlantic salmon (Howard & Carson 1991) and from some biofouling communities on salmon seacages (Tan et al. 2002). It is not yet known if there is a link between strains of *Neoparamoeba* sp. isolated from non-host environments, such as sediments and biofouling communities, and AGD. The aim of this study was to identify *Neoparamoeba* sp. in the sediments at sites within 2 salmon farming leases and to determine whether any relationship or pattern exists between the presence of the organism and coexisting environmental factors. Environmental variables included in the study were: redox potential, sulphide concentrations and general biological condition of the sediments immediately under fish pens, and background farm data such as freshwater bathing events or fallowing state.

## MATERIALS AND METHODS

**Farm leases.** Sediment samples and geochemical measures were taken from 4 sites at 2 separate farm leases in south eastern Tasmania (Fig. 1). At Farm 1 (Creeses Mistake) the 4 sites included 2 reference positions outside the lease boundary, 150 m from the nearest pen, and 2 sites directly beside 2 of the fish pens. At Farm 2 (Stringers Cove) 4 sites adjacent to fish pens were sampled. At Farm 1 samples were collected in May, August and February 2002 and March 2003, whilst at Farm 2 samples were collected in February, July and August 2002 and February 2003. Farm 1 is more exposed with predominately fine sand sediments, whereas Farm 2 is a sheltered site with finer sediments dominated by silt/clays.

**Sample collection and *Neoparamoeba* culture and identification.** Marine sediment samples were collected in duplicate at each farm on 3 different occasions, using a Van Veen Grab sediment sampler with a 0.0675 $m^2$ surface area. Sub-samples of between 200 to 800 g were taken from the top few cm of sediment for analysis, and attempts were made to culture *Neoparamoeba* sp. from this material using techniques described by Page (1983). The sediments were first transported back to the laboratory for processing within 24 h. In the laboratory the samples were then shaken for 1 h to enable thorough mixing. A 3 to 5 g aliquot was then spread onto malt yeast seawater (MYS) agar plates (0.1 g malt, 0.1 g yeast, 750 ml filtered seawater, 250 ml reverse osmosis water, 250 µl pimaricin as an antifungal agent) which had been seeded with *Stenotrophomonas maltophilia* or *Escherichia coli* as a food organism. The MYS plates were then incubated at 20°C and, on the appearance of amoebae cells were sub-cultured to fresh MYS plates until there were enough trophozoites present for harvest and identification. *Neoparamoeba* sp. was identified among populations of amoebae based on the existence of a parasome, an endosymbiont (Dyková et al. 2003), reactivity with a polyclonal antibody visualised by an immunofluorescent antibody test (Howard & Carson 1993) and detection, via polymerase chain reaction (PCR), of small subunit (SSU) rDNA originally thought to be specific to *N. pemaquidensis* (Wong et al. 2004) but subsequently also found capable of detecting *N. branchiphila*. Sites were designated positive if one or both duplicate sediment samples revealed the pres-

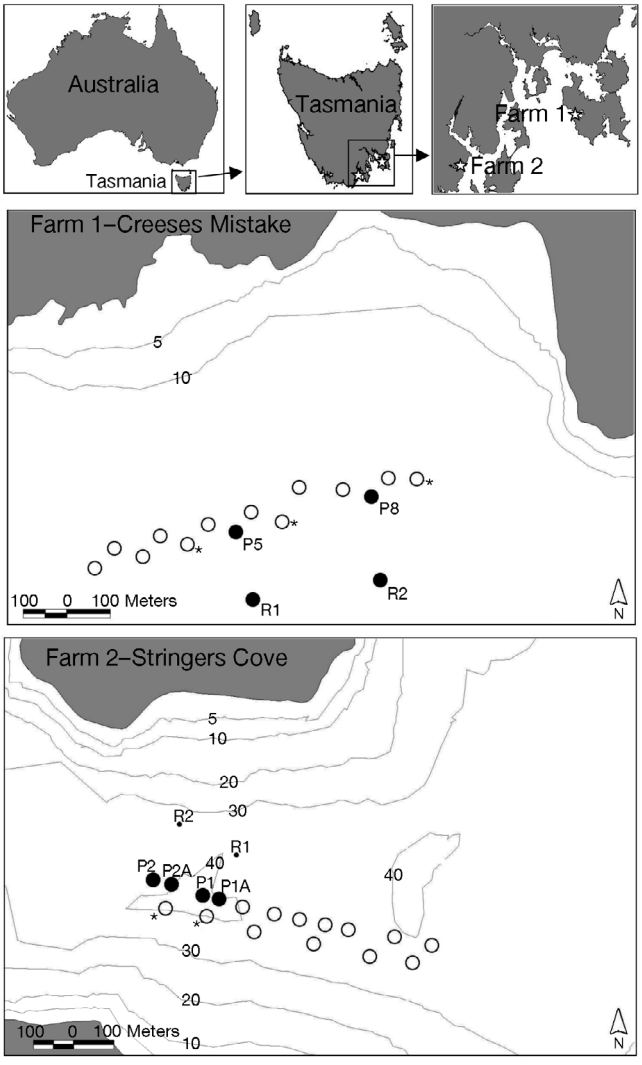


Fig. 1. Atlantic salmon pen positions for sediment samples at Farm 1 and Farm 2 in south eastern Tasmania. Farm 1 (Creeses Mistake) is located in Wedge Bay on the Tasman Peninsula and Farm 2 (Stringers Cove) is within Port Esperance. Sample pen sites (●) = R1, R2, P5 and P8 for Farm 1 and P2, P2A, P1 and P1A for Farm 2. Other pens (○) remained unstocked whilst adjacent pens (○*) werestocked during the sample periods

ence of *Neoparamoeba* sp., negative if *Neoparamoeba* sp. was not detected in cultured populations of amoebae or if no amoebae were isolated by culture. The results are presented as the percentage of replicates showing positive for *Neoparamoeba* sp.

**Site reduction/oxidation (redox) potential and sulphide assessment.** Three replicate cores (perspex tubes 250 mm long × 45 mm internal diameter) were taken for measurement of redox potential and sulphide concentration at each farm lease. A Craib corer was used to collect core samples at Farm 1 and a specially designed multicorer (Macleod et al. 2004) was used at Farm 2. Both redox potential and sulphide concentration were measured at 3 cm. Redox was measured using a WTW Redox Probe whilst sulphide measurements were collected using a Cole-Parmer 27502-40 silver/sulphide electrode according to the method described by Wildish et al. (1999).

**Visual assessment of sediments.** Video footage was captured using a digital underwater camera system linked by an umbilical to a digital recorder on the surface with a minimum of 2 min footage recorded from each sample site. Images were scored according to key features determined to be indicative of marine farming-impacted or non-impacted conditions, as defined by previous research (Crawford et al. 2001, Macleod et al. 2002). Each impact feature was weighted according to its sensitivity to enable us to detect impacted or non-impacted sites, and scores for each were designated negative or positive, respectively (Macleod et al. 2004).

**Statistical analyses.** The relationship between the presence of *Neoparamoeba* sp. and individual environmental variables at each site was examined by regression analysis with an $\alpha$ value of 0.05, using Microsoft Excel. There were no regression analyses for references sites 1 and 2 at Farm 1 as data sets were incomplete.

Table 1. *Neoparamoeba* sp. Presence (+) or absence (–) of *N.* sp. in duplicate marine sediment samples from 4 sites each within 2 Atlantic salmon farming leases in Tasmania. n.a. = no amoebae recovered

| **Farm 1** Site | R1 (Reference site) | R2 (Reference site) | P5 | P8 |
|---|---|---|---|---|
| Mar 02 | +/– (50%) | No samples | +/– (50%) | No samples |
| Aug 02 | –/ n.a. (0) | No samples | –/n.a. (0) | –/n.a.(0) |
| Jan 03 | +/+ (100%) | +/n.a. (50%) | +/– (50%) | +/n.a. (50%) |
| Mar 03 | –/– (0) | +/n.a. (50%) | n.a./n.a. (0) | +/+ (100%) |
| **Farm 2** Site | P1 | P2 | P1A | P2A |
| Feb 02 | +/+ (100%) | +/+ (100%) | +/+ (100%) | +/+ (100%) |
| July 02 | +/n.a. (50%) | +/n.a. (50%) | +/n.a. (50%) | +/– (50%) |
| Aug 02 | +/+ (100%) | +/+ (100%) | +/+ (100%) | +/n.a. (50%) |
| Feb 03 | n.a./n.a. (0) | –/n.a. (0) | –/– (0) | –/n.a. (0) |

## RESULTS

With the exception of 1 site on each farm, amoebae were successfully isolated from sediments at all sampling sites on both farming leases at each time (Table 1), with 72.4 % of all sediment samples yielding viable amoebae after a period in culture. Furthermore, *Neoparamoeba* sp. was consistently isolated from Farms 1 and 2 at all times except for the final sample periods in February and March 2003, respectively. Across both leases, 42 out of 58 sediment samples yielded amoebae, of which 29 were shown to include *Neoparamoeba* sp. At Farm 2 duplicates at all sites were positive for *Neoparamoeba* sp. in February 2002, whereas in February 2003 all sites were negative. At these times either there were no amoebae recovered from the sediments or those that were isolated were not *Neoparamoeba* sp. Sediment samples were not available for amoebae culture from some sites at Farm 1 in March and August 2002. In this study we have assumed a relationship between the ability to detect *Neoparamoeba* sp. within a given sample and its abundance; therefore, a positive result for duplicates at any site indicates an abundance of the amoeba.

The stocking and freshwater bathing regimes within individual pens appear to have no influence on the presence of *Neoparamoeba* sp. in the respective sediments. Sample sites at Farm 2 in 2002 consistently yielded *Neoparamoeba* sp. irrespective of whether individual pens were stocked or fallow. Sampling time lines (Fig. 2) show recent stocking/ fallowing history and freshwater bathing events for each site. Freshwater bathing events may have an impact on sediment populations of *Neoparamoeba* sp. as they have the potential to return viable amoebae to the environment.

Regression analyses showed no relationship between *Neoparamoeba* sp. presence and environmental factors. *Neoparamoeba* sp. was present regardless of fluctuations in redox potential, sulphide concentrations or the observed benthic impact resulting from fish farming (Figs. 3 & 4). There was no evidence of organic enrichment from fish farming at the Farm 1 reference positions. Video scores for the reference sites were positive at all times, sediment redox potentials were relatively high and sulphide measurements were low, which suggest an oxic and non-reducing environment. However, these sites consistently indicated the presence of *Neoparamoeba* sp. over the sampling period. Unfortunately, there were no equivalent samples taken from reference sites at Farm 2.

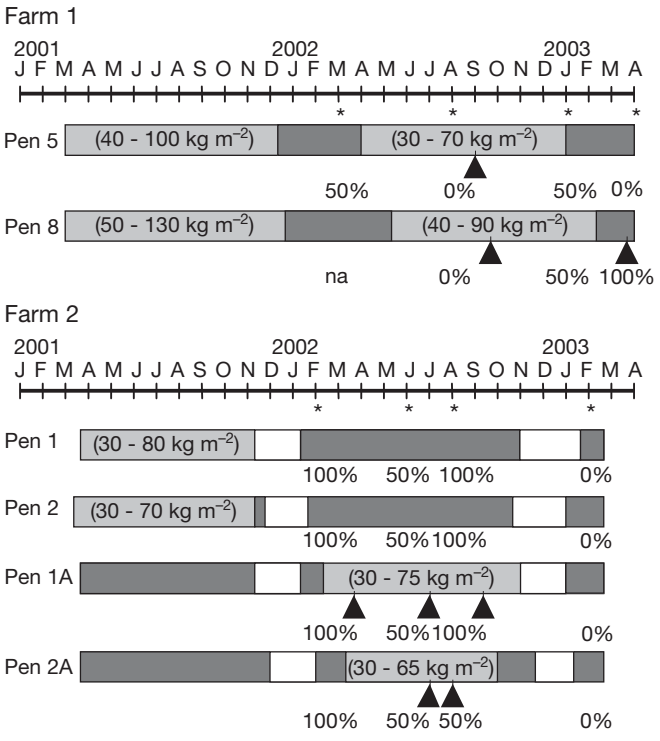


Fig. 2. *Neoparamoeba* sp. Timeline showing when sediment samples were collected (*) and percentage of replicates positive for *Neoparamoeba* sp., stocking status (□ cage stocked, ■ cage empty and □ lease empty) biomass range (kg m$^{-2}$; biomass expressed as kg per area of the sediment directly beneath respective sea cages) and freshwater bathing event (▲) for individual pens (P): P5 and P8 at Farm 1 and P1, P2, P1A and P2A at Farm 2 from early 2001 to February/April 2003. Note: Although at times the study cages were empty, adjacent pens (20 to 30 m away) may have been stocked. Lease empty means that there were no stocked pens on the lease

There was evidence of a seasonal effect at Farm 1 during the winter sampling (August 2002) when no *Neoparamoeba* sp. was recovered, whereas at Farm 2 a similar result was found only in the final summer sampling point (February 2003).

## DISCUSSION

This study further supports the findings of Crosbie et al. (2003) which suggest that *Neoparamoeba* sp. is widely distributed in Tasmanian marine sediments. Members of the *Neoparamoeba* genus are common (Page 1983) and have been shown to occur in a diverse range of habitats including degraded and organically enriched environments. *Neoparamoeba* sp. was recovered by culture using sediment sample sizes comparable to those used in the present study (less than 5 g) from a variety of conditions, including clean Atlantic Ocean sediments, chemical dump sites, off-shore sewage disposal sites and areas under oil platforms (Sawyer 1980). In the above study, *Paramoeba pemaquidensis* (the genus has since been renamed *Neoparamoeba*) was recovered from all of the study sites, including those most impacted by oil contamination in the Gulf of Mexico. The present results suggest that *Neoparamoeba* sp. is both abundant and widespread in the sediments around fish farms, as the amoeba was recovered using very small amounts of sediment (3 to 5 g) from 50 % of samples.

It was not possible to identify any specific relationship between the incidence or occurrence of *Neoparamoeba* sp. and the intensity or location of farming operations. The wide environmental tolerances of this organism make it extremely difficult to distinguish any relationship between the sediment conditions associated with salmon farming and the presence of *Neoparamoeba* sp. in the environment. The relationship between the incidence of AGD, the presence of *Neoparamoeba* sp. on salmon and the abundance of the amoeba in the environment is also complex. Although freshwater bathing at the sites may decrease *Neoparamoeba* sp. infection of salmon, as evident by the reduced gill scores, it may in turn disproportionately load the sediments with the amoeba. Freshwater bathing was used at the sites studied for fish displaying various levels of severity of gill infection (data not shown) but on most occasions it occurred 2 or more mo before the sediment sampling. The fate of *Neoparamoeba* sp. once shed into the bathing water is not known.

There was some evidence suggesting a seasonal influence on *Neoparamoeba* sp. abundance, but this was different at each farm. All sites sampled at Farm 1 were negative for *Neoparamoeba* sp. in the winter (July) of 2002. However, at Farm 2 no *Neoparamoeba* sp. were recovered from the sediments in the summer (Feb) of 2003 from any of the sample sites although they were detected here on all other occasions, including the previous summer (Feb 2002). Not detecting *Neoparamoeba* sp. during the summer is unusual as this is a time when AGD is more prevalent due to the higher water temperatures (Clark & Nowak 1999) and possibly greater numbers of the organism. There were no unusual fluctuations in temperature or salinity during either summer (data not shown) which could explain this result. Inability to detect the amoeba in the sediments during this period may indicate a lack of sensitivity of the enriched culture recovery method. It should be noted that the amoebae recovery method used for this study has some limitations; in particular, there may be some risk of false negatives as a result of the small amount of sediment inoculum. Furthermore, disparity between results for duplicates from some

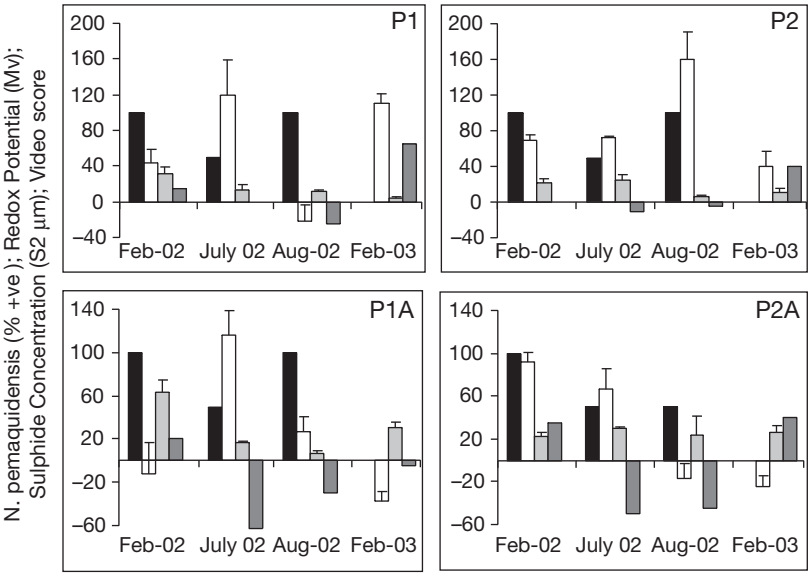


Fig. 3. *Neoparamoeba pemaquidensis.* Percentage of duplicate sediment samples yielding: ■ *N. pemaquidensis*, together with □ redox potential, ▒ sulphide concentrations (both at 3 cm depth), and ▩ assessment of benthos conditions based on video observations, for 4 sites within the Atlantic salmon farming lease at Stringers Cove (Farm 2). For redox and sulphide concentrations values are means +SE (n = 3)

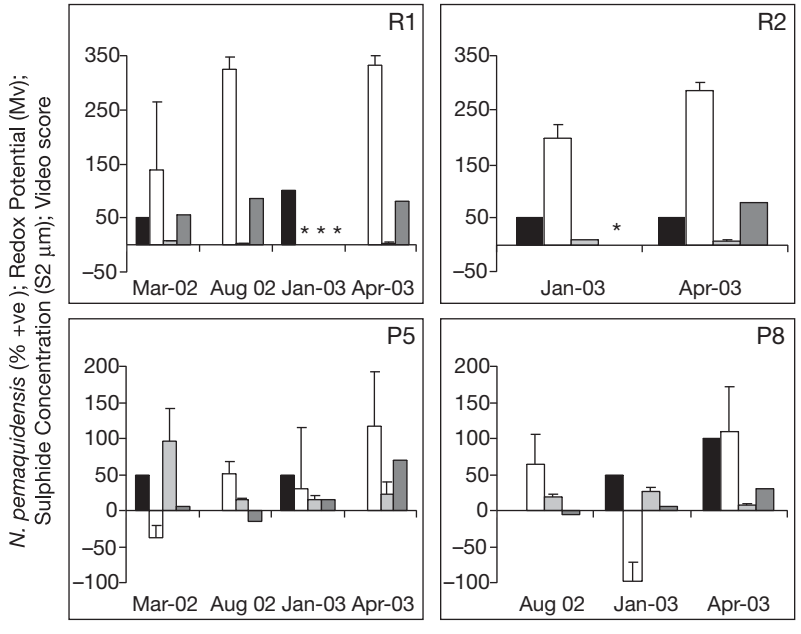


Fig. 4. *Neoparamoeba pemaquidensis.* Percentage of duplicate sediment samples yielding: ■ *N. pemaquidensis*, together with □ redox potential, ▒ sulphide concentrations (both at 3 cm depth), and ▩ assessment of benthos conditions based on video observations, for 4 sites within the Atlantic salmon farming lease at Cresses Mistake (Farm 1). There were no sediment samples available for amoebae isolation at Reference site 2 (R2) during Mar and Aug 02 nor at site P8 in Mar 02; therefore data are not included for these periods. *Data not collected include redox potential, sulphide concentrations and video scores for Reference site 1 (R1) in Jan 03 and video scores for R2 in Jan 03. For redox and sulphide concentrations values are means +SE (n = 3)

sites supports the notion of a lack of sensitivity due to natural small scale variability. However, there were only 2 occasions where no amoebae at all were recovered from either duplicate and these occurred at one site on each farm.

A major limitation to discerning a relationship between salmon farming and *Neoparamoeba* sp. in the environment was the inability to quantify the amoeba in either the sediments or the water column. Although in many ecological studies of amoebae diversity and abundance enumeration occurs indirectly after a culture step (Rogerson & Hauer 2002), amoebae are distinguished by morphotype (Butler & Rogerson 1995, Anderson 1998, Anderson et al. 2001) rather than by genus or species. Attempts were made to culture and recover amoebae from water samples after concentration by filtration, but these were unsuccessful. In a previous study by Douglas-Helders et al. (2003), where *Paramoeba/Neoparamoeba* sp. was detected in the water column by immuno-dot blot and quantified using the most probable number, the highest densities were found within the fish cages and a reduction was seen with distance from the cages. However, these methods could not be applied in the current study as they do not distinguish between viable and non-viable cells; furthermore, the antibody used for the immuno-dot blot may be cross-reactive with other environmental (non-fish host) organisms (Douglas-Helders et al. 2003). The inability to quantitatively evaluate *Neoparamoeba* sp. in the sediments has also limited the extent to which we were able determine the significance of farm practices in the distribution and prevalence of the organism.

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