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Temporal and spatial distribution of paramoebae in the water column – a pilot study

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Abstract

Amoebic gill disease is the main disease affecting the salmonid industry in Tasmania, but no information on the distribution of the causative pathogen, *Neoparamoeba pemaquidensis*, in the aquatic environment is available. This pilot study aimed to determine temporal and spatial distributions of paramoebae species in the water column, using an immuno-dot blot technique. Water samples were collected from inside fish cages at various depths (0.5, 5.5 and 11.0 m) in both summer and winter, as well as various distances (0, 0.5, 240, 280, 750 and 1100 m) away from the sea cage and farming site. Paramoebae densities were estimated using the most probable number technique (MPN). Temperature, salinity, dissolved oxygen, turbidity, nitrite and nitrates, and bacterial counts were measured for each water sample. Data were analysed using a residual maximum likelihood test and significant associations between paramoebae densities and environmental factors were analysed. Results showed that densities were significantly higher in summer ($P = 0.017$), at 5.5 m depth ($P = 0.029$), and reduced to the lowest density at 1100 m away from the cage sites ($P = 0.008$). Bacterial counts, turbidity and temperature were found to be significantly associated with paramoebae densities.

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Introduction

Amoebas are extremely abundant in the marine environment and have been collected from inshore areas, throughout the oceanic water column, as well as from sediments (Bovee & Sawyer 1979; Sawyer 1980). For example, 76 species of marine amoebae from the waters of the north-eastern United States were described by Bovee & Sawyer (1979). Most marine amoebae are bactivorous (Bovee & Sawyer 1979; Anderson 1988; Paniagua, Parama, Iglesias, Sanmartin & Leiro 2001), although some are also known to feed on other protozoans, algae or organic detritus (Bovee & Sawyer 1979; Sawyer 1980; Page 1983). Marine amoebae were shown to be affected by season, which in turn was correlated to water temperature, and by dominance and competition among different amoeba species (Anderson 1988). Water temperature, salinity and the availability of food were suggested to be major factors affecting amoeba distributions (Bovee & Sawyer 1979). Aquatic organisms show highest growth and survival at optimum growth conditions (Rheinheimer 1974). Thus, growth *in vitro* of *Neoparamoeba pemaquidensis* was enhanced at temperatures above 5 °C (Kent, Sawyer & Hedrick 1988), with an upper limit of 22 °C (Howard 2001). Optimum growth of this protozoan was seen at 15‰ salinity, with little decline in growth rate up to 30‰ salinity (Kent *et al.* 1988).

Six species from the genus *Paramoeba* were described by Kent *et al.* (1988), including *P. aestuarina* Page, *P. pemaquidensis* Page (now known as *N. pemaquidensis* Page), *P. eilhardi* Schaudinn, *P. schaudinni* de Faria, *P. perniciosa* Sprague and *P. invadens* Jones. Of these, *P. perniciosa* was pathogenic for the blue crab, *Callinectes sapidus* Rathbun, (Sprague, Beckett & Sawyer 1969) and *P. invadens* was pathogenic for sea urchin, *Strongylocentrotus droebachiensis* Müller (Jones 1985). *N. pemaquidensis* was found to be pathogenic for salmonids, turbot, *Scophthalmus maximus* (L.), European sea bass, *Dicentrarchus labrax* (L.), and sharpsnout seabream, *Diplodus puntazzo* Cuvier (Kent *et al.* 1988; Roubal, Lester & Foster 1989; Munday, Foster, Roubal & Lester 1990; Clark & Nowak 1999; Dykova, Figueras & Peric 2000; Kent 2000; Dykova & Novoa 2001). *N. pemaquidensis* is thought to be an amphizoic (Scholz 1999) or opportunistic protozoan (Kent *et al.* 1988), which means that the normally free-living protozoan becomes pathogenic under certain conditions (Scholz 1999).

Amoebic gill disease (AGD) is the main disease affecting the salmonid industry in Tasmania. Amoebic gill disease is caused by the naked and lobose protozoan *N. pemaquidensis* (Page 1983). It is unable to form cysts and does not have flagella (Bovee & Sawyer 1979). Although some epidemiological studies have been reported (Douglas-Helders, Nowak, Zilberg & Carson 2000; Douglas-Helders, Saksida, Raverty & Nowak 2001a; Douglas-Helders, Dawson, Carson & Nowak 2002; Douglas-Helders, Weir, O'Brien, Carson & Nowak, unpublished data), to date no information on the spatial or temporal distribution of the pathogen in the water column is available. *N. pemaquidensis* was first detected in the water column from marine waters off Maine, USA (Page 1970) and is the most common marine amoeba (Page 1983), with a worldwide distribution (Cann & Page 1982). The protozoan is often found in coastal waters and the lower reaches of estuaries (Page 1983). *N. pemaquidensis* has also been detected in heavily polluted waters, including those contaminated by heavy metals (Sawyer 1980).

This pilot study provides the first estimation of the spatial and temporal distribution of paramoebae in and around a Tasmanian salmon farm, and provisionally relates the distribution to environmental conditions, thus providing an insight into the ecology of AGD, which will help determine future research, control and monitoring programmes.

Materials and Methods

Validation and sensitivity of detection methods for paramoebae in water

A gill isolate of *Paramoeba* sp. was obtained from a known AGD infected Atlantic salmon donor fish, which originated from AGD infected stocks, held in an experimental tank at 13 °C and at a salinity of 37‰. The donor fish was anaesthetized using 100 mg L^{-1} of benzocaine and the paramoebae isolated as described by Zilberg, Gross & Munday (2001). The isolate was washed twice with 0.45 μm sterile (121 °C, 15 min) and filtered sea water (SFS) by centrifugation at 2600 ***g*** for 15 min. The pellet was resuspended in 10 mL SFS and a viable cell count performed using 0.5% trypan blue and a haemocytometer (Zilberg *et al.* 2001). Triplicate dilutions were made, with final paramoebae cell numbers of 1000, 100, 10 and 1 in 1 mL of SFS. SFS without paramoebae was used as a negative control. From all tubes an 80 μL aliquot was used for testing with immuno-dot blot as described by Douglas-Helders, Carson, Howard & Nowak (2001b), including the digestion and cell lysis steps.

Water samples were taken from various locations in Tasmania (Table 1), to determine if paramoebae

Table 1 Sources, replicates, and number of samples taken at different depths and in multiple volumes from each site for field validation

Region	Source	*n*	Sampling depth (m)	Total volume sampled (mL)	Test volume (μL)
East coast	Bicheno 1	3	0–0.5	100	80, 200, 400
Tasmania	Bicheno 2	6	0–0.5	50	80, 200, 400
North coast Tasmania	Tamar freshwater	2	0–0.5	50	800
	Tamar mouth	32	0–0.5	50	800
Southeast coast	Hideaway Bay	36	0, 5, 10	2000	80, 240, 320
Tasmania	Garden Island	36	0, 5, 10	2000	80, 240, 320
	Tinderbox	4	0–0.5	2000	80, 240

species could be detected in the aquatic environment and to validate testing using the immuno-dot blot technique. Samples were taken from two salmon farms in the Huon Estuary, South-East Tasmania, at three different sites. Two of these sites contained infected salmon, while the other site was fallow. Water samples were also taken from the east coast of Tasmania, more than 100 km away from any salmon farming sites, and from the mouth of the Tamar river in the north of Tasmania, with one salmon farm approximately 20 km away. This farm was known to be free from AGD. Turbid freshwater samples were taken upstream from the Tamar River to assess the effect of organic particles in the sample. Water samples were stored on ice until processed in the laboratory. Sample volumes of 100, 50 and 0.24 mL were concentrated to 800 μL by centrifugation, and 800 μL of the water sample was left unconcentrated. From these, volumes of 80, 160, 200, 240 and 320 μL were inoculated onto the test membrane to determine the minimum test volume to provide a positive dot blot result. All water samples were processed and analysed as described by Douglas-Helders *et al.* (2001b), with the exclusion of the mucus digestion step. In brief, water samples were concentrated by centrifugation and treated with 40 μL of 0.21% v/v sodium hypochlorite and 0.045% v/v sodium hydroxide. After 8 min of incubation the samples were further treated by adding 10 μL of 2 N hydrochloric acid, and incubated for a further 30 min. Finally, the samples were frozen at −20 °C, defrosted just prior to analysis, and centrifuged for 20 s at 15 600 ***g***. The supernatant was used for blotting onto a prewetted PVDF membrane (Bedford, MA, USA), using a 96 well vacuum dot-blotter (Millipore). After 18 min incubation, the membrane was washed four times using PBS and PBS with 0.05% Tween 20 added (PBS-T), blocked using 2.5% w/v casein (BDH, cat. no. 44016) in PBS-T and again washed four times. Prepared membranes were probed with a polyclonal anti-PA027 primary antibody (Douglas-Helders *et al.* 2001b), incubated for 25 min and washed as described, followed by incubation with a secondary alkaline phosphatase conjugated anti-rabbit antibody (Silenus, Melbourne, Australia) for 20 min and subsequent washing steps. The blots were visualised using fast BCIP/NBT (Moss Inc., Pasadena, MD, USA) and colour development stopped by washing the membrane twice for 5 min in reagent grade water. Best visualization was obtained when the membrane was still wet and all tests were read at this stage. SFS and PBS enriched with *N. pemaquidensis* PA027 (DPIWE) were used as positive controls, while un-lysed natural seawater samples were used as a control for the lysis process, and SFS as well as PBS were used for negative controls. Nine of the water samples were tested for the presence of *N. pemaquidensis* using nested PCR (Elliott, Wong & Carson 2001).

Distribution

The AGD prevalence status on the farm sites at the time of sampling was estimated using the farm's own gross gill lesion scoring system. White mucoid patches or excessive mucus are an indication of AGD infection and can range from small, light spot-like discolorations affecting one or two gill lamellae, to more visible mucus build up, but with only a very small area of the gill affected, to larger areas of affected gills with clearly seen white patches. The severity of AGD infection in a cage was based on the number of fish examined, usually between 20 and 30 fish, and the degree of infection for each fish. This resulted either in a light, medium or heavy score for the cage. Water sampling took place on two different sites at one farm in the Huon Estuary, SE Tasmania. The water samples were taken using a 5 L Niskin bottle (General Oceanics Inc., Miami, FL, USA), connected to a rope with clearly marked 1-m intervals. The paramoebae distribution in sea cages at different depths and seasons was determined by duplicate water sampling of two cages from three different depths, in both summer and winter. Duplicate water samples were also taken at 5.5 m depth from inside sea cages and at 0.5, 240, 280, 750 and 1100 m away from these cages to determine the spatial distribution. The first sampling point outside the cage was chosen to determine the reduction of paramoebae because of the cage net, while the last sampling point was the maximum distance that could be covered before the tide changed. Paramoebae densities in water samples were quantified using the most probable number technique (MPN) (Oblinger & Koburger 1975; Gonzalez 1996). For this, five sub-samples of 400, 200, 100, 50 and 25 mL for each water sample, were concentrated to 800 μL by centrifugation. The resulting 25 sub-samples for each water sample were tested for the presence of paramoebae using the immuno-dot blot technique described previously. The number of

dot blot positive sub-samples for each of the five concentrations resulted in five numbers, each between zero and five. These numbers represented the most probable number (MPN) of paramoebae per litre, using the custom-made MPN table based on the program described by Gonzalez (1996).

Seasonal and depth distribution of paramoebae in cages

Duplicate water samples were taken from two sea cages, from slack tide to an ebb tide, both in summer (February 2002) and winter (August 2001). Samples were taken from 0.5, 5.5 and 11.0 m depths, which represented the surface, middle and bottom of the sea cage. Winter sampling took place on the Hideaway Bay site in the Huon Estuary, SE Tasmania. In summer, no cages were present at this site and water samples were taken from the Garden Island site, in close proximity to the Hideaway Bay site. The Garden Island site was positioned approximately 2.5 km NE from the Hideaway Bay site, in an estuary that runs from NW to SE. Thus, between season comparisons have to be interpreted with caution. However, the study is concerned with the distribution of paramoebae from a known source such as an infected cage, rather than from the same geographical position.

Spatial distribution of paramoebae

Duplicate water samples were taken in summer (February 2002) from two sea cages at the Garden Island site. Sampling took place on two consecutive days when the greatest difference between high and low tides occurred, and at the start of the ebb tide. All water samples were taken from 5.5 m or mid-cage depth, where paramoebae densities were presumed to be high, as determined during the validation samplings. Firstly, duplicate water samples were taken from the centre of the sea cage (0 m sample), and from just outside the cage (0.5 m sample). In an attempt to follow the same water mass, two current measuring devices (CMD) or drogues were placed into the water column at 3 m (CDM_3) and 6 m (CDM_6), respectively (Fig. 1). The CMDs were constructed from an empty two litre, sealed plastic bottle as the floating device, to which a 3-m (CMD_3) or 6-m (CMD_6) length rope (3 mm polyethylene) was attached. At the end of the rope, two circular pieces of plastic (4 mm polyvinyl sheet) were attached at 90 degrees to each other (Fig. 1). The CMD was weighted using a 0.5 kg lead weight. Once the CMDs were placed into the water column, their position was regularly monitored using a global positioning system (Differential GPS, Garmin GPSMAP 135 receiver/sounder with attached Ausnav, Aztec RXMAR 1; Aus Navigation, Bentleigh East, Victoria, Australia). The GPS monitored distance from the point of origin, total travel distance, and current position. At each sampling point the global position was recorded, the Niskin bottle lowered into the appropriate sampling position, and a 5 L sample taken. Then, the boat was returned to within 3 m of the same sampling point, using the GPS, and the Niskin bottle lowered down again for a duplicate 5 L water sample. The water samples taken in this manner could be considered replicates, however, more drogues would have been required to accurately represent the water flow with time. This was not possible because of sampling logistics. At 240 and 280 m from the point of origin, water samples were taken following the CMD_6, while at 750 and 1100 m samples were taken following the CMD_3, as a result of the faster travelling speed of the latter. This was necessary to enable sampling at greater distances from the farm site before the turn of the tide, which would not have been possible using the CMD_6 only.

Environmental measurements

Environmental factors such as temperature, salinity and dissolved oxygen were measured for each water sample. The number of bacteria in the seawater samples was estimated using a protocol developed by the Fish Health Unit, Department of Primary Industries, Water and Environment, Launceston, Australia. A sterile (121 °C, 15 min) 30 mL container was submerged into the water sample, the screw cap removed and the cap replaced underwater to avoid contact with the surface layer. Five replicate Johnson's marine agar (JMA) (Johnson 1968) plates per water sample were inoculated with 50 μL of the 30 mL sample, spread with sterile hockey stick spreaders (Oxoid, Melbourne, Australia), using a different spreader for each plate. The plates were stored on ice during transport and placed into a 20 °C incubator (Kelvinator 380; Labec Pty Ltd, Marrickville, NSW, Australia) for 24 h, then incubated at 15 °C (Thermoline, Selby, Australia) for 48 h,

Figure 1 A current measuring device (CMD) or drogue, which was used for tracking a water mass to determine the spatial distribution of paramoebae in the water column.

after which the number of colony forming units was counted. The viable bacterial count per mL was determined only for plates with counts of 10–300 colonies, to avoid an unacceptably large degree of error. Turbidity was determined in triplicate for each water sample using a 2100P turbidity meter (Hach Company, CO, USA) set at auto range and signal averaging. The turbidity was expressed in nephelometric turbidity units (NTU). The comparisons of turbidity measurements and total microbial counts were used to draw conclusions on the type of substances responsible for turbidity (Rheinheimer 1974). A positive correlation between turbidity and bacterial counts meant that the turbidity was because of an increase in the amount of suspended organic matter (Rheinheimer 1974). Dissolved nutrients (nitrites and nitrates in N-μg L^{-1}) were measured by the APHA Method 4500, carried out by the NATA Accredited Analytical Services (Tasmania Laboratories, Hobart).

Statistical analysis

Distribution data were analysed using the residual maximum likelihood (REML) technique (Patterson & Thompson 1971), using the software package Genstat version 4.2, fifth edition (VSN International Ltd, Oxford, UK). This test estimates the treatment effects and variance components in a linear mixed model. This technique was used instead of ANOVA because in this situation the data were unbalanced. The REML analysis produces a Wald statistic, which is analogous to the F-statistic in ANOVA. Wald statistics have an approximately chi-squared distribution and are evaluated in terms of chi-squared probabilities for the degrees of freedom associated with particular fixed effects. The response variate was the MPN or the estimate of paramoeba numbers; the fixed factors were depth, season, distance and the interaction of depth and season. Replicates (cage depth and seasonal distribution), sample, and sample replicates (spatial distribution) were fitted into the random model. Correlation coefficients of the assessment for association between environmental factors and MPN estimates were calculated using Genstat version 4.2 (VSN International Ltd).

Results

Validation and sensitivity

The consistent sensitivity of the immuno-dot blot was ten paramoeba cells per millilitre of SFS when testing water samples, while all SFS samples tested dot blot negative. Of the nine seawater samples tested with both immuno-dot blot and nested PCR, four samples were positive for dot blot while no *N. pemaquidensis* could be detected using PCR (Elliott *et al.* 2001). The results of the field samples showed that all East coast samples and Tamar River fresh and seawater samples were negative for the presence of paramoebae, while samples from salmonid farms in the Huon Estuary tested positive, depending on the sample volume used (Table 2). Paramoeba presence could be detected when a

Table 2 Results of immuno-dot blot tests for paramoebae using different sample volumes and different test volumes of field water samples from Atlantic salmon farming sites

Region	Sample volume (mL)	Test volume (μL)	Dot blot results for each sample and replicate
Hideaway Bay 0 m	100, 50, 0.240, 0.08	80 and 240	100: 100% weak pos. 50, 0.24, 0.08: 100% neg
Hideaway Bay 5 m	100, 50, 0.240, 0.08	80 and 240	100, 50: 100% pos. 0.24, 0.08: 100% neg.
Hideaway Bay 10 m	100, 50, 0.240, 0.08	80 and 240	100: 100% weak pos. 50, 0.24, 0.08: 100% neg.
Garden Island 0 m	100, 50, 0.240, 0.08	80 and 240	100: 100% pos. 50: 100% weak pos. 0.24, 0.08: 100% neg.
Garden Island 5 m	100, 50, 0.240, 0.08	80 and 240	100: 100% pos. 50: 100% weak pos. 0.24, 0.08: 100% neg.
Garden Island 10 m	100, 50, 0.240, 0.08	80 and 240	100: 75% weak pos. 50: 100% weak pos. 0.24, 0.08: 100% neg.
Tinderbox 1	100, 50	160	100: 100% pos. 50: 100% neg.
Tinderbox 2	100, 50	160	100: 100% pos. 50: 100% neg.

Neg: negative, weak pos.: weak positive, pos.: positive.

sample volume of 100 mL, and occasionally 50 mL, was used, but not in volumes of 240 and 80 μL. The volume applied to the dot blot membrane did not affect the test result.

Distribution

The cages sampled to determine the cage depth and seasonal distribution were all heavily infected with AGD, according to farm records. The site from which the spatial distribution was determined was medium to heavily infected with AGD at the time of sampling. Within sea cages, the highest density of paramoebae was found at 5.5 m depth ($P = 0.029$, df2, Wald stat 7.06; Fig. 2) and densities were significantly higher in February compared with those in August ($P = 0.017$, df1, Wald stat. 5.69; Fig. 2). Significantly lower paramoebae densities were found at 240 and 1100 m from the sea cage ($P = 0.008$, df5, Wald stat. 15.75; Fig. 3). At 1100 m the CMD was outside the site area, at approximately 750 m from the southernmost sea cage. The CMDs took 259 min to travel 240 m, 94 min for 280 m, 71 min for 750 m and 276 min for 1100 m. This represented travel speeds of 92.7 (CMD_6, day 1), 297.8 (CMD_6, day 2), 1056.3 (CMD_3, day 2) and 398.5 (CMD_3, day 1) cm s^{-1}. Averages of the environmental conditions during sampling are shown in Table 3.

A significant association was found between paramoebae densities and the number of bacteria, both for the cage depth and temporal study ($r = 0.841$, $P < 0.01$), and the spatial study ($r = 0.807$, $P < 0.01$). Temperature was positively correlated with paramoebae densities ($r = 0.431$, $P < 0.05$) in the cage depth and temporal study, while turbidity was positively correlated in the spatial study ($r = 0.549$, $P < 0.05$). In addition, there was a positive correlation between temperature and numbers of bacteria in the water column in the cage depth and temporal distribution study ($r = 0.439$, $P < 0.05$), as well as between bacterial numbers and turbidity in the spatial study ($r = 0.695$, $P < 0.01$). When correlation analysis was performed for the different depths, significant correlations were found between paramoebae densities and temperature ($r = 0.784$, $P < 0.05$), salinity ($r = -0.792$, $P < 0.05$), and

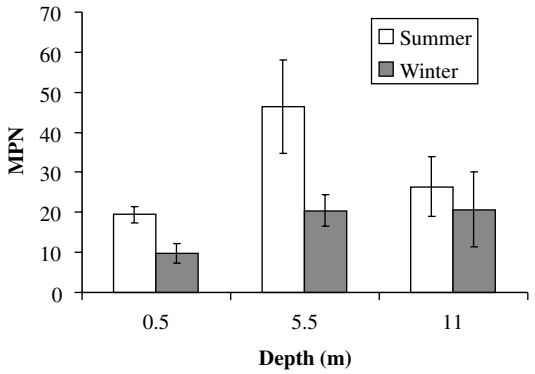

Figure 2 Temporal and spatial paramoebae distributions in sea cages on a medium to heavily AGD infected farming site.

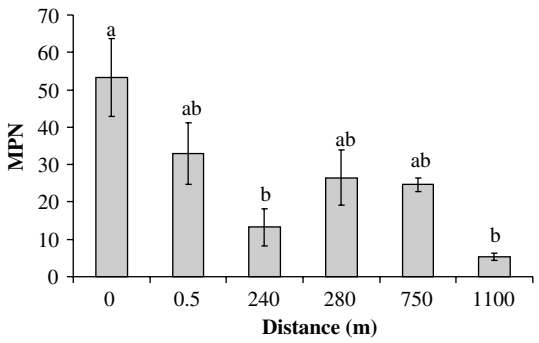

Figure 3 Spatial distribution of paramoebae at 5.5 m depth from 0 m (in cage) and up to 1100 m away from the cages.

Table 3 Averages of environmental conditions (SD) during water sampling for spatial and temporal studies

Environmental variable		Spatial study	Cage depth and temporal study	
		Summer	Summer	Winter
Salinity	0.5 m		28.7 (0.3)	34.4 (0.1)
‰	5.5 m	34.1 (0.5)	33.7 (0.5)	34.5 (0.1)
	11.0m		34.6 (0.2)	34.6 (0.1)
Temperature	0.5 m		14.3 (0.2)	12.5 (0.1)
(°C)	5.5 m	16.2 (0.3)	15.3 (0.1)	12.5 (0.0)
	11.0 m		15.5 (0.2)	12.5 (0.0)
Bacterial counts	0.5 m		459.3 (111.2)	445.8 (319.8)
(cfu)	5.5 m	1239.9 (1386.6)	740.3 (268.9)	692.0 (272.8)
	11.0 m		1910.0 (1118.5)	1015.0 (373.4)
Nitrite & nitrate	0.5 m		8.0 (1.41)	33.5 (2.9)
($\mu g\ L^{-1}$)	5.5 m	NM*	5.75 (1.3)	33.0 (2.3)
	11.0 m		11.5 (3.4)	34.5 (0.6)
Turbidity	0.5 m		1.57 (0.11)	1.80 (0.41)
(NTU)	5.5 m	1.37 (0.4)	1.02 (0.10)	3.00 (0.43)
	11.0 m		1.00 (0.14)	2.48 (1.34)

*Not measured.

dissolved nutrients ($r = -0.807$, $P < 0.05$) at the surface. At 5.5 m and 11 m the only significant correlation found was between paramoebae density and bacterial numbers in the water column.

Discussion

Paramoebae densities were highest in summer, at 5.5 m depth inside sea cages, and densities reduced away from the farming site. Average seawater temperatures in the summer are higher than in winter, affecting a range of biological factors in the water column. Increasing temperatures, within the organism's viable range, promotes biological reactions, such as an increase in bacterial populations because of seasonal temperature fluctuations (Rheinheimer 1974). In the spring and summer algal blooms are more likely to occur because of the increased temperature and longer daylight hours. Jelly fish, including possibly harmful species, can also be more numerous at this time of the year. In addition, the dissolved oxygen level in the water column decreases at higher temperatures. Thus, as a result of the complexity of the field situation, causal relationships between environmental factors, pathogen presence and AGD can be difficult to determine without laboratory experiments. The significant positive correlation between paramoebae densities and temperature observed in this study suggests that temperature may be a causal factor in AGD, which supports similar conclusions drawn in previous studies (Clark & Nowak 1999; Douglas-Helders *et al.* 2001a; Munday, Zilberg & Findlay 2001; Nowak 2001). A significant correlation between paramoebae and the number of bacteria in the water column was also found in this study, as well as a significant correlation between temperature and number of bacteria. While both temperature and bacterial counts are possible risk factors in outbreaks of AGD, either or both of these factors might be confounding (Thrusfield 1995). However, at 5.5 and 11 m depth, where paramoebae densities were higher compared with 0.5 m depth, bacterial count was the only variable significantly correlated with paramoebae densities. Kent *et al.* (1988) suggested that other factors than temperature, such as abundance of food organisms in the water column, could promote paramoebae blooms. This suggests that bacterial counts could be a causal risk factor for AGD infections. Controlled trials are required to resolve these issues of interrelation.

In this study the highest paramoebae densities were found at 5.5 m depth inside sea cages. The fluctuations of environmental factors such as salinity, temperature and dissolved oxygen are greatest at the surface layer and may negatively affect paramoebae densities. At deeper levels, the physical environment is more stable and fluctuations are narrower. This may be a more suitable environment for a naked and lobose protozoan (Cann & Page 1982) that is known to be unable to form cysts (Bovee & Sawyer 1979) for surviving unfavourable conditions. The high paramoebae density observed at 5.5 m was perhaps because of the greater abundance of available hosts at that depth. The vertical distribution of Atlantic salmon in sea cages varies with seasonal and diurnal rhythms (Fernö, Huse, Juell & Bjordal 1995). Fish tend to avoid the

surface because of light intensity, as well as the bottom of sea cages (Fernö *et al.* 1995). Extensive studies are needed to fully understand which factors determine paramoebae density at mid sea cage depth.

Densities of paramoebae generally decreased with increasing distance from the cage. The lowest density was found furthest from the sea cages and outside the farming site. This could be the result of paramoebae attaching to solid surfaces such as nets or hosts while in transport with the water flow, or merely through dilution, thus creating a washout effect of protozoan numbers with distance travelled from the cage. In previous studies *N. pemaquidensis* has been found on nets (Tan, Nowak & Hodson 2002) and attachment to fish gills is well known (Roubal *et al.* 1989; Munday *et al.* 1990; Adams & Nowak 2001). The significant correlation of paramoebae densities with turbidity and bacterial numbers, and the significant correlation between bacterial numbers and turbidity, suggests that these factors are interrelated. A strong association between bacterial numbers and turbidity was also found in a study of the water column in the western Baltic (Rheinheimer 1974). The relationship between paramoebae densities and turbidity was not found in the cage depth and temporal study, but sampling always took place within a sea cage, and turbidity is likely to be cage-dependent. As a positive relationship was found between turbidity and bacterial numbers, it was concluded that the turbidity was the result of the amount of suspended organic matter (Rheinheimer 1974). It is not unlikely that particles in the water column play an important part as a vector in the transmission process of paramoebae to fish. Controlled laboratory experiments are needed to determine the causality of bacterial densities, turbidity and total suspended organic particles, for AGD occurrence.

Detection of paramoebae using immuno-dot blot was successful, although no PCR positive water samples could be detected. The test sensitivities of immuno-dot blot and nested PCR are different, with the dot blot being able to detect 10 cells in one mL while PCR only detects 16 cells in 100 μL (Elliott *et al.* 2001). The sensitivity results for the two tests were both based on detection of cultured *N. pemaquidensis* in 0.45 μm filtered sea water. It is possible that the water sampled in this study contained certain PCR inhibitors resulting in a decreased sensitivity of the test. Tests are currently under way to resolve this issue and thus increase the sensitivity of the PCR. However, the MPN results of this study are still only just within the lowest detection limit of the current PCR sensitivity limit (M. Douglas-Helders & P. Crosbie, unpublished results). In the case of environmental isolates, the immuno-dot blot detected a pool of near related paramoebae species (Douglas-Helders *et al.* 2001b), which could increase the sensitivity of the test. The antibody cross-reacted with *N. aestuarina* Page and *Pseudoparamoeba pagei* Sawyer, but not with *Paramoeba eilhardi* Schaudinn (Douglas Helders *et al.* 2001b). Although *P. pagei* has been detected in waters of similar temperature as Tasmanian waters (Page 1983), *P. eilhardi* has only been found in the Mediterranean and Indian Ocean (Page 1983), making it less likely to be present in Tasmanian waters. No cross reactivity was detected with *Platyamoeba plurinucleolus* Page, *Platyamoeba/Vanella* Page or *Flabellula* Schmoller (DPIWE FLB 004) (Howard & Carson 1993). Paramoebae densities were possibly overestimated in this study because of cross-reactivity of the primary antibody. However, all control samples taken away from AGD positive farms tested dot blot negative, including the 100 mL samples, suggesting that any overestimation was minimal. Production of a monoclonal antibody would be required to detect *N. pemaquidensis* only. However, Dykova *et al.* (2000) suggested that more refined diagnostic methods would be needed before *N. aestuarina*, one of the cross-reactive species in the immuno-dot blot test, could be excluded as a possible agent of AGD as well as *N. pemaquidensis*. Future studies are needed to resolve the involvement of other amoebae species in AGD.

We report here preliminary results on the distribution of paramoebae in the water column. Larger scale investigations are necessary to clarify seasonal effects and to determine the relationship between paramoebae densities and bacterial counts and turbidity.

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