Notes

Isolation of *Neoparamoeba pemaquidensis* Page, 1987 from Marine and Estuarine Sediments in Tasmania

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Abstract

To gauge environmental ubiquity of *Neoparamoeba pemaquidensis,* the causative agent of amoebic gill disease (AGD) in farmed Atlantic salmon in Tasmania, sediments in both marine and estuarine sites, including those with no history of AGD, were screened for presence of the organism. With one exception *N. pemaquidensis* was detected in all locations.

Neoparamoeba pemaquidensis Page, 1987 is the causative agent of amoebic gill disease (AGD) in finfish mariculture (Nowak et al, 2002). The disease can have a significant impact on Atlantic salmon production in Tasmania (Munday et al., 1990, Munday et al., 1993, Clark and Nowak, 1999). However, the environmental distribution of this pathogen in Tasmania is not fully understood. This amoeba has been isolated and cultured from the marine environment in many parts of the world and is thought to be one of the more common marine amoebae (Page, 1983). However, in Tasmania *N. pemaquidensis* has only been isolated and cultured from gills of AGD-affected Atlantic salmon (Howard and Carson, 1991) and from some biofouling communities on salmon seacages (Tan et al., 2001). The amoeba has also been detected, but not isolated, in seawater using immunodotblot (Douglas-Helders et al., 2003). The aim of this study was to determine the presence of *N. pemaquidensis* in sediments at marine and estuarine sites around Tasmania including some reference sites with no history of finfish culture.

Marine sediment samples were collected from various sites within specific areas around Tasmania and attempts made to culture *N. pemaquidensis* from this material. Areas comprised finfish culture and non-culture sites, these included both estuarine and oceanic locations. The most extensively sampled sites were in the Huon Estuary and on the Tasman Peninsula where samples were collected on 3 and 2 occasions respectively. Other sites, which were sampled once were: Hideaway Bay, Bruny Island, Tinderbox, Tamar estuary, Bicheno and Macquarie Harbour (Figure 1). Sediment sample volumes ranged from approximately 200-800 g. Isolation of amoebae

from sediments was attempted using techniques described by Page (1983). Briefly, 3-5 g of sediment material was smeared 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) which had been seeded with *Stenotrophomonas maltophilia* as a food organism, then incubated at 20ºC and examined every 5-6 days.

When sufficient numbers of amoebae were cultured 3 identification methods were employed to confirm the presence of *N. pemaquidensis*. Firstly, the amoebae were harvested from the MYS plates using sterile seawater, a small volume of the suspension was then placed onto a slide and allowed to dry before an immunofluorescent antibody test (IFAT) (Howard and Carson, 1993) was performed to detect *N. pemaquidensis*. Secondly, another proportion of the suspension was used as a wet slide preparation for observation of the characteristic parasome using either differential interference contrast (DIC) microscopy or after staining with 4'6-diamino-2-phenylindole (DAPI) to highlight the nuclei and parasomes. The staining protocol with DAPI was adapted from that of Howard (2001), briefly 200-500 µL of the cell suspension was incubated with formalin (37% formaldehyde) to a final concentration of 3% v/v and 10-25 µL of DAPI solution (0.05 mg per mL reverse osmosis water) for at least 30 mins in the dark. After incubation a wet mount of the suspension was prepared and examined with a fluorescent microscope with a filter block in the UV excitation range. Thirdly, the final proportion of the harvested cell suspension was subjected to a DNA extraction procedure (Wilson and Carson, 2001)

then a polymerase chain reaction (PCR) using *N. pemaquidensis*-specific primers of the 18S rDNA gene sequence (Elliott et al., 2000). Presence of *N. pemaquidensis* was believed confirmed when a positive result was recorded for all three detection methods.

In virtually all cases the sediments yielded amoebae within 7-14 days and *N. pemaquidensis* was shown to be present in sediments from all areas (Table 1), including those where there is no history of AGD in farmed salmonids and where salinities fluctuate (i.e. Tamar estuary and Macquarie Harbour). The only site where *N. pemaquidensis* was not detected was the in-shore reference site at Macquarie Harbour. It is therefore reasonable to conclude that this amoeba is ubiquitous in the marine environment in Tasmania. It is also of interest to note that sediment types ranged from sand to much finer, denser, organically-rich and anoxic material. The isolation by culture indicates not only the presence of *N. pemaquidensis* in marine sediments but also its viability.

Area	Date	n	Positive (%)	AGD status
Stringer's Cove, farm	19/02/02	12*	75	Positive
	02/07/02	12*	58	
	04/08/02	12*	58	
Nubeena, farm	19/03/02	4*	50	positive
	10/09/02	6*	0	
Hideaway Bay, farm	19/04/02	5	20	Positive
Tamar Estuary	23/04/02	4	25	Negative
Tinderbox, farm	09/05/02	4	25	Positive
Bruny Island, farm	27/05/02	4	75	Positive
Macquarie Harbour, farm	17/10/02	9	11	Negative
Bicheno, non- finfish farm site	07/04/02	4	50	Negative
Macquarie Harbour, reference site	06/05/02	5	0	Negative

Table 1. Results of *N. pemaquidensis* detection in sediments sampled from various sites around Tasmania and their amoebic gill disease (AGD) status. *At these farms samples were supplied in duplicate, therefore n = 12 refers to duplicate samples from 6 sites within the farm, similarly n=4 or 6 means 2 or 3 sites within the farm.

The fact that many sediment samples did not yield *N. pemaquidensis* is thought to be a sensitivity issue and there is also a reasonable possibility of false negative results as *N. pemaquidensis* can be difficult to culture (I. Dyková pers com). It is likely that the amoeba is more common in Tasmania than these results would indicate and a lack of detection does not necessarily imply absence. Even though there was no attempt made to concentrate any amoebae which may be in the sediments and only a small amount of sediment was used to inoculate the MYS plates a significant proportion of sediments nevertheless yielded *N. pemaquidensis*.

This study indicates that, as in other parts of the world, *N. pemaquidensis* is a common marine amoeba in the Tasmanian coastal environment. The relationship between presence of *N. pemaquidensis* and disease outbreaks is not clear and the virulence of the sediment strains is not known. Quantitative detection methods would need to be used to investigate the relationship between *N. pemaquidensis* density and AGD occurence.

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