A CELL CULTURE SYSTEM FOR PARAMOEBIASIS RESEARCH

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ABSTRACT

Paramoebiasis caused by the amphizoic amoeba *Neoparamoeba pemaquidensis* is a significant disease in various commercially important finfish and invertebrates worldwide. Outbreaks of paramoebiasis have recently become more severe and are not longer restricted to the warmer months of the year. The lack of understanding of the biology of this parasite is reflected by the unavailability of effective drugs in invertebrates while an increased resistance to treatment in finfish is observed. Its loss of virulence on artificial media and the inability to obtain axenically large numbers of pathogenic parasites are major limitations to many areas of paramoebiasis research. Recently, it was shown that *N. pemaquidensis* grows rapidly on a rainbow trout gill cell line and is cytopathogenic to the monolayer. This thesis further investigated this newly established method of culture. First, its advantages and limitations were compared to conventional maintenance methods of *N. pemaquidensis*; so far, this is the only method that yields a pure and dense culture of amoebae at a reasonable cost. Secondly, the potential recovery of the initial pathogenicity of *N. pemaquidensis* towards two susceptible salmonid hosts was explored via transmission studies. Using immersion with a high concentration of amoebae, Amoebic Gill Disease could not be induced in Alantic salmon or rainbow trout. Finally, the suitability of this source of amoebae for *in vitro* experiments was tested by developing a reproducible and fast method of screening various treatments (freshwater, lasalocid, oxytetracycline, Virkon®) using flow cytometry (FCM). Although the results indicate that *N. pemaquidensis* grown on rainbow trout gill cells are not good organisms for chemotherapeutant screening, FCM remains a useful method for evaluation of antiparasitic agents.

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DEDICATION

A Salomon et Annette

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LIST OF ABBREVIATIONS

°C	degree Celsius
18S rRNA	Gene coding for the small subunit of the ribosome in Eukaryotes
ADP	Adenosine Di-Phosphate
AGD	Amoebic Gill Disease
ARB	Amoeba Resistant Bacteria
ASW	Artificial Sea Water
ATCC	American Type Culture Collection
AVC	Atlantic Veterinary College
AVCLSC	Atlantic Veterinary College Lobster Science Centre
CCAC	Canadian Council on Animal Care
CCAP	Culture Collection of Algae and Protozoa
CPE	Cytopathic Effect
CSA	Cerophyll Sea water Agar
CTDEP	Connecticut Department of Environmental Protection
d	day
Da	Genetic distance between strains: number of net nucleotide substitutions per site between strains
DFO	Fisheries and Oceans Canada
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
ELIS	Eastern Long Island Sound
FBS	Foetal Bovine serum
FCM	Flow Cytometry
FL	Fluorescence
FSC	Forward scatter
FW	Freshwater
g	gram
G	Centrifugal force

GAE	Granulomatous Amoebic Encephalitis
h	hour
IFAT	Indirect Fluorescent Antibody Test
IRO	*Ichthyobodo necator* Related Organism
L	Liter
L-15	Leibovitz medium
LC_{50}	Lethal Concentration 50 %
LIS	Long Island Sound
m	meter
M2	Dead fluorescent cells
min	minute
Osm	Osmole
MTS	3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	[3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
n	number
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NBT	Nitro-blue tetrazolium
OD	Optical Density
OTC	Oxytetracycline
PAM	Primary Amoebic Meningoencephalitis
PCBs	Polychlorobiphenols
PCR	Polymerase Chain Reaction
PES	Phenazine ethosulfate
PI	Propidium Iodide
PIT	Passive Integrated Transponder
R1	Total cell count
rRNA	ribosomal ribonucleic acid
s	second
SD	Standard deviation

SEM	Scanning Electron Microscopy
Sp.	species
SSC	Side scatter
SSU	small subunit
t	metric tonne
TEM	Transmission Electron Microscopy
UI	International Units
USA	United States of America
v	volume
WLIS	Western Long Island Sound
XTT	3-bis-(2-methoxy-4-nitro- 5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

I. GENERAL INTRODUCTION

1.1 Impact of Paramoebiasis

Page created the genus *Neoparamoeba* in 1987 for some marine amoebae (amoeba, from the Greek "amoibe" meaning "change") previously classified in the genus *Paramoeba*, Schaudinn 1896. *Paramoeba* and *Neoparamoeba* are genera of small, naked amoebae with lobose (finger-like) pseudopodia (Cann & Page 1982). A classification scheme for Eukaryotes has recently been proposed (Adl et al. 2005). The system is based on nameless ranked systematics and incorporates both ultrastructural and molecular phylogenetic studies. Based on this system, *Paramoeba* and *Neoparamoeba* spp. are included in Amoebozoa: Flabellinea: Dactylopodida. *Paramoeba and Neoparamoeba* spp. are amphizoic as they may be free living or parasitic (Culbertson et al. 1958). Some have caused sporadic mass mortalities of populations of echinoderms and crustaceans on the east coast of North America since the 1970s, with drastic economic and ecological impacts as described below. Paramoebiasis has also been a major disease in finfish aquaculture, especially in Australia (Munday 1986).

1.1.1 Epidemic Mass Mortalities in Marine Invertebrates

1.1.1.1 "Gray Crab Disease" of Blue Crabs

In Virginia (USA), unknown amoeboid cells containing two nucleus-like bodies were discovered in dead or moribund blue crabs, *Callinectes sapidus*, by Sprague and Beckett (1966) (Figure 1.0).

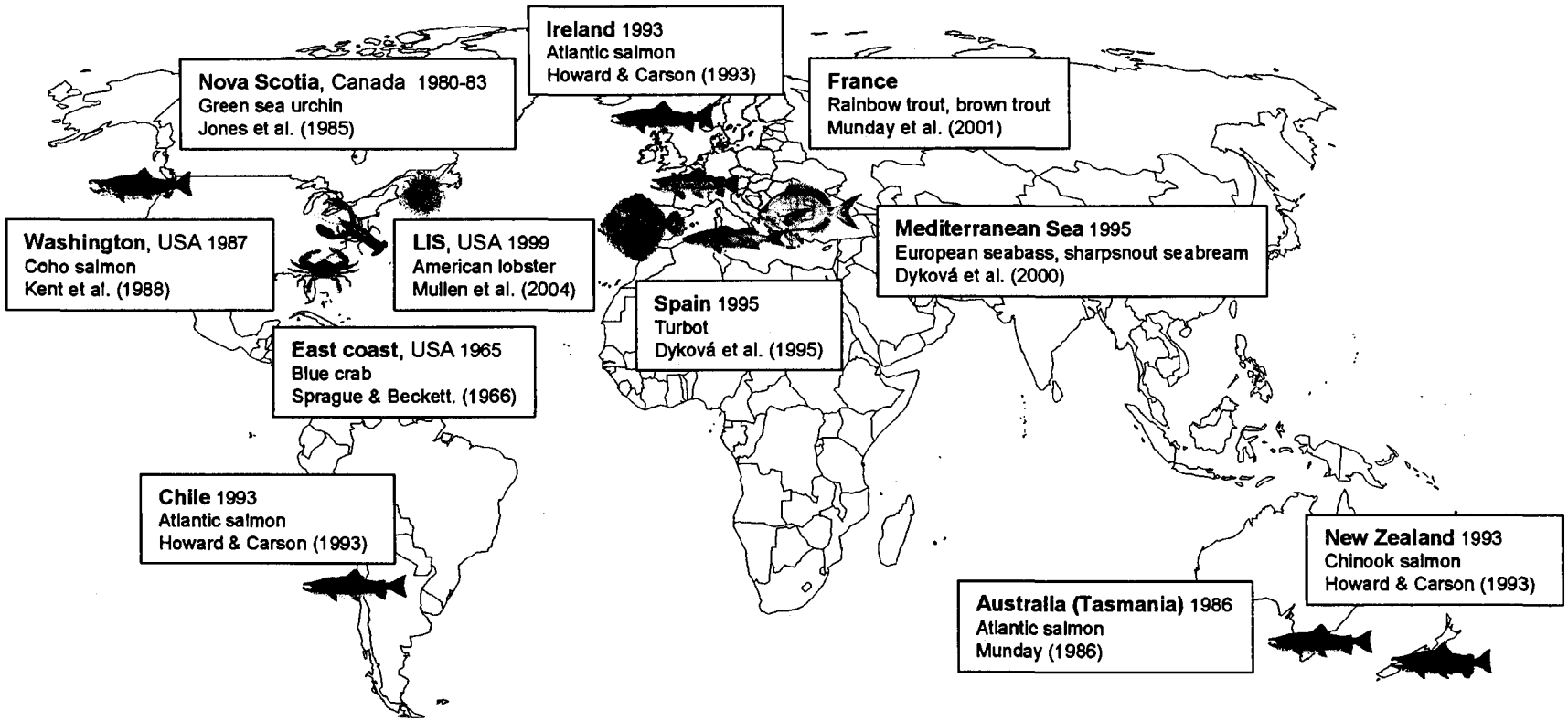

Figure 1.0. Worldwide distribution of paramoebiasis. *Neoparamoeba* spp. have been reported from all continents, except Africa and Asia, in cultured marine finfish and wild invertebrates. This genus of amphizoic amoeba has also been isolated as a free-living organism from the marine environment. LIS: Long Island Sound. Adapted from Caraguel and used with permission.

The abdomens of diseased crustaceans were gray and their appendages contained cloudy hemolymph and watery tissue. Sick individuals were sluggish and died soon after removal from water. The disease was named by Sprague & Beckett (1966) after these clinical manifestations, i.e. "Gray Crab Disease" and was significant because of the economic value of these crustaceans. The first suggestion was that the amoeboid cells were crab cells infected with a virus (Sprague & Beckett 1966). The nature of the etiological agent of "Gray crab disease' was later determined by Sprague & Beckett (1968) by comparing these cells with *Paramoeba eilhardi*, a free-living amoeba found initially in a public aquarium in Berlin (Germany). Based on its failure to survive in common culture media for marine amoebae, its small size, and its finger-like pseudopodia, the name *Paramoeba perniciosa* was proposed, to recognize its pathogenicity for *C. sapidus* (see Sprague et al. 1969).

In heavy infections of blue crab, the connective tissues, blood sinuses and closed vessels were filled with amoebae. Extensive damage to the hematopoietic tissues, Y organ, and supporting connective tissues was observed, and pre-mortem heart necrosis developed (Johnson 1977). Lysis occurred in connective tissue, hemocytes, epidermal cells and hematopoietic tissues while lysis of skeletal muscle was reported terminally (Johnson 1977). Fat storage in the tubule epithelium of the hepatopancreas was reduced in terminal infections (Johnson 1977). Hemolymph smears often showed only parasites and sometimes large amounts of lysed muscle tissue (Sprague et al. 1969). Hemocytes were rare or absent (Sawyer 1969).

Reappearance of the parasite, coinciding with significant crab mortalities lasting for 30 days, occurred in early summer when sea temperatures were 18-30 °C (Sawyer et

al. 1970, Newman & Ward 1973). Occasional light infections were found in hibernating crabs (Sawyer et al. 1970, Newman & Ward 1973). Johnson (1977) detected the presence of *P. perniciosa* in crab tissues during all seasons, suggesting that the winter carryover of the parasites occurred in the crustaceans (Johnson 1977).

Coincident with some of the crab die-off, Mirex, an extremely stable and persistent organochlorine insecticide (half life up to 10 years) was aerially applied as part of a fire ant control program in the gulf of Mexico (Newman & Ward 1973). However, the impact of pesticides on crab mortalities is unknown (Newman & Ward 1973).

1.1.1.2 Paramoebiasis of Green Sea Urchins

In the early 1980s (Scheibling & Stephenson 1984, Miller 1985, Scheibling 1986) and 1990s (Scheibling & Hennigar 1997), recurrent outbreaks of paramoebiasis resulted in mass mortalities of green sea urchins, *Strongylocentrotus droebachiensis*, in the subtidal zone of the Atlantic coast of Nova Scotia, Canada. Sea urchin mortalities were estimated to be 245,000 t from 1980 to 1983 (DFO 1994). Clinical signs of moribund echinoderms included loss of attachment to the substratum, inability to extend tube feet, dropping spines, and gaping peristome and jaw followed by progressive epidermal necrosis (Jones et al. 1985). Li et al. (1982) found that gonadal and digestive tracts contained an amoeboid protist, classified at that time as *Labyrinthomyxa* sp. In 1985, Jones et al. isolated an amoeba from sections of tissues from moribund sea urchins in laboratory and field populations. The presence of a second nucleus-like structure led to the naming of this amoeba as *Paramoeba invadens* (see Jones 1985). Experiments

were then conducted *in vivo* and confirmed the involvement of this protozoan in the pathogenesis of sea urchin disease (Jones & Scheibling 1985).

Experimental induction of paramoebiasis in sea urchins showed that development of clinical illness was temperature-dependent: signs rapidly occurred above 16 °C (10 days) compared to 12 °C (20 days) (Scheibling & Stephenson 1984). Progression of the disease slowed when water temperature decreased, and stopped between 8 and 12 °C (Scheibling & Hennigar 1997). In the ocean, temperature played a crucial role in outbreaks as mass mortalities were most notable in the fall, for a 2-3 month period, especially in years when unusually high seasonal peak water temperatures (16-18 °C) occurred (Scheibling & Hennigar 1997).

1.1.2 Chronic Amoebic Gill Disease in Finfish

The free living amoeba *Neoparamoeba pemaquidensis*, first identified by Kent et al. (1988) as *Paramoeba pemaquidensis,* was recently re-classified as *Neoparamoeba pemaquidensis* by Dyková et al. (2000). *N. pemaquidensis* causes Amoebic Gill Disease (AGD) in several cultured marine fish including Atlantic salmon, *Salmo salar*, Coho salmon, *Oncorhynchus kisutch* (see Kent et al. 1988), Chinook salmon, *Oncorhynchus tshawytscha* (see Howard & Carson 1993), and rainbow trout, *Oncorhynchus mykiss* (see Munday et al. 2001). It also affects turbot, *Scophtalmus maximus* (see Dykovà et al. 1995), European seabass, *Dicentrarchus labrax* (see Dyková et al. 2000), and sharpsnout seabream, *Diplodus puntazzo* (see Dyková et al. 2000). Amoebic gill disease is the only infectious disease seriously threatening Atlantic salmon mariculture in Tasmania, Australia (Munday 1986, Findlay & Munday 1998, Clark & Nowak 1999)

and New Zealand (Howard & Carson 1993). This disease has also been reported from farmed Atlantic salmon in Ireland (Howard & Carson 1993, Rodger & McArdle 1996, Palmer et al. 1997), France (Munday et al. 2001), Spain (Dyková et al. 1995), Chile (Howard & Carson 1993), and the west coast of USA (Kent et al. 1988).

Infected fish are lethargic and crowd together at the water surface with flared opercula. In Atlantic salmon, gross signs of infection include slightly raised white mucous patches on the gills and excessive mucus covering the entire body, whereas in rainbow trout the mucoid patches appear relatively diffuse and increased mucous secretion is confined to the gills (Munday et al. 2001, Roberts & Powell 2005). Histologically, gill hyperplasia is observed, generally resulting in fusion of the secondary lamellae (Kent et al. 1988). Fusion of the distal portion of secondary lamellae results in the formation of large interlamellar vesicles or crypts and is coincident with the presence of hypertrophied epithelial cells (Roubal et al. 1989, Dyková et al. 1998, Clark & Nowak 1999). Amoebae are typically confined to the gill surface and rarely penetrate the epithelium (Zilberg & Munday 2000, Adams & Nowak 2003) but may become entrapped within interlamellar vesicles (Kent et al. 1988, Munday et al. 1993, Dyková et al.1995, Adams & Nowak 2001, Parsons et al. 2001). No histopathological changes in visceral organs are associated with amoeba infestations (Kent et al. 1988).

The pathophysiology of AGD is still uncertain. As infected fish have respiratory difficulties, initially the cause of death was suggested to be asphyxiation with gill lesions and extensive mucous secretion leading to reduced gill surface area. Oxygen uptake is reduced and gas transfer across the gills is impaired (Adams & Nowak 2001, Clark & Nowak 1999, Dyková et al. 1998). However, Powell et al. (2000) demonstrated

that clinical AGD has moderate impact on the respiratory status of salmon and further suggested fish were dying because of cardiac failure (Powell et al. 2002a, 2002b). Extracellular acidosis appears to be the first metabolic disturbance and is associated with vascular hypertension, increasing vascular resistance and morphological changes in the ventricle. Consequently, there is cardiac dysfunction leading ultimately to death (Powell et al. 2002a, 2002b).

Research on AGD has focused on environmental and host risk factors associated with the disease. Long-term infections in salmonids have been associated with full or near-full strength sea water (32 ‰) (Clark & Nowak 1999, Munday et al. 1990, Rodger & McArdle 1996). Although *Neoparamoeba* sp. has been observed at 7.2 ‰ salinity (Clark & Nowak 1999), it generally grows poorly in salinities lower than 10 ‰ (Kent et al. 1988). In turbot, AGD has occurred at 22 ‰ salinity (Dyková et al. 1998). The discrepancy in environmental salinity for disease onset in turbot versus other fish species may reflect the presence of the recently described *N. branchiphila* (Dyková et al. 2005). Therefore, it is plausible that *Neoparamoeba* spp. have adapted preferentially to different hosts and salinities.

Water chemistry plays an important role in amoeba survival. Powell & Clark (2003) investigated the effects of divalent cations on the viability of gill-harvested amoebae *in vitro* and found that high concentrations of Ca^{2+} and Mg^{2+} (200 mg/ L) promote prolonged survival of the marine *Neoparamoeba pemaquidensis* in freshwater. Consequently, the current treatment of AGD-affected fish with a freshwater bath for 2-4 h could be improved by the use of soft freshwater (19.3-37.4 mg $CaCO_3$/ L) rather than hard freshwater (173-236.3 mg $CaCO_3$/ L) (Roberts & Powell 2003).

Amoebic gill disease has not yet been reported in countries with cold water aquaculture species such as Canada, Scotland, Iceland and Norway. Generally, AGD in Atlantic salmon occurs at water temperatures of 12-20 °C (Munday et al. 1990, Rodger & McArdle 1996). In turbot, AGD was observed at 14-18.8 °C (Dyková et al. 1998). In Tasmania, amoebae were observed on the gills of Atlantic salmon during the winter with no clinical signs or histological lesions, thus suggesting that the temperature could be the main factor triggering the disease (Munday et al. 1990). However, recent outbreaks in Atlantic salmon have occurred at 9.1-10.6 °C in Australia (Clark & Nowak 1999, Douglas-Helders et al. 2001a).

Some of the host factors influencing the severity of outbreaks include fish age, size and species. Atlantic salmon appears to be the most susceptible salmonid to AGD and smolt is the most sensitive stage to the infection (Nowak 2000). Reinfection occurs more slowly in mature fish (larger gills, better immunity?) but very large fish are more sensitive to reinfection (poorer gill condition?). Although fish develop anti-paramoeba immunoglobulins after both natural or experimental infections, it appears unlikely that these antibodies elicit any specific protection against AGD (Nowak 2000). Additionally, triploid salmon, which form an important part of farm stocks, apparently display greater effects from infection (decreased growth and higher mortality rates) (Nowak 2000).

Amoebic gill disease costs represent about 10 % of the gross value of farmed salmon mariculture in Tasmania but may cost up to 20 % of the production (Munday et al. 2001). Additional to fish losses, the current treatment, oxygenated freshwater baths (exposure to salinity lower than 4 ‰ for 2 to 4 h) repeated every 4-6 weeks during

summer months, is time consuming and expensive. Moreover, the necessity for a farm to have a good quality fresh water supply (low total hardness) and the difficulties of administering freshwater baths limit the success of salmon farming. This treatment is effective against AGD for only up to three weeks (Clark & Nowak 1999), probably because *N. pemaquidensis* survive in the crypts formed by fusion of secondary lamellae (Parsons et al. 2001). Amoebic gill disease makes salmon aquaculture non viable in Spain (J. Arán personal communication cited by Munday et al. 2001). Although the economic impact of AGD worldwide is not well documented, there is significant potential for mortality and morbidity caused by *Neoparamoeba* spp. to be a barrier to successful salmonid aquaculture.

1.1.3 Acute Die Off of American Lobsters

In the fall of 1999, mass mortality of American lobsters, *Homarus americanus*, occurred in western Long Island Sound (WLIS) (Figure 1.1). Increased numbers of dead and moribund sea urchins, starfish and various crab species were also reported in LIS (CTDEP 2000). It was estimated that 11 million lobsters died, resulting in the collapse of the entire lobster fishery (Mullen et al. 2004). William M. Daley, the U.S. Secretary of Commerce declared the lobster fishery a "commercial failure due to a resource disaster" in early 2000 (CTDEP 2000). Federal funds (US $13.9 million) were provided to support lobstermen and researchers investigating the causes of the mortality (Pearce & Balcom 2005).

Post-mortem examinations of dead and moribund lobsters were performed to determine the putative cause of the die off.

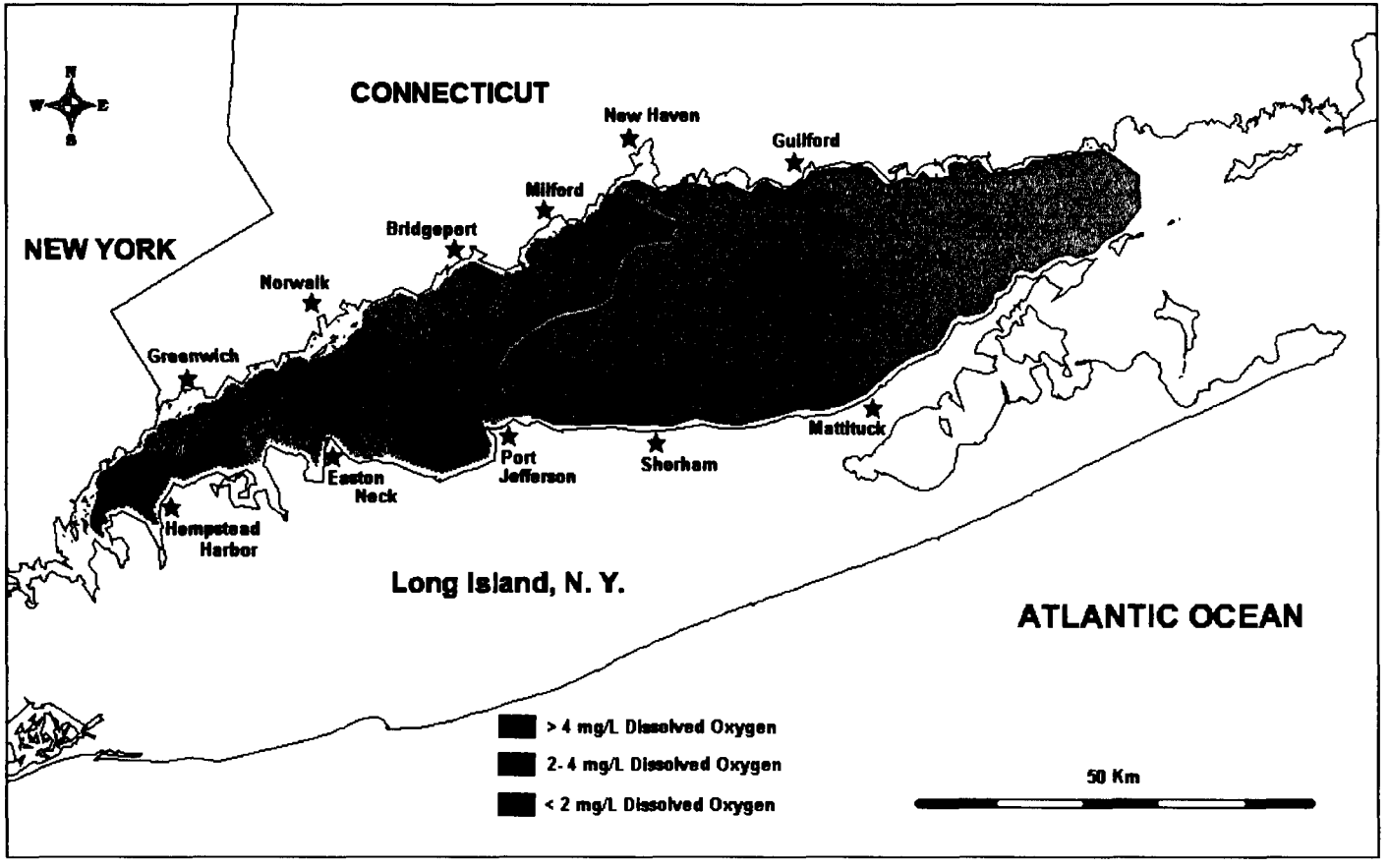

Figure 1.1. Dissolved oxygen levels in Long Island Sound. The Sound is arbitrarily split into Western Long Island Sound (WLIS) and Eastern Long Island Sound (ELIS). The shade panels represent areas where the concentration of dissolved oxygen was determined between August 2-5, 1999 (adapted from CTDEP Long Island Sound monitoring website: http://www.ct.gov/dep/cwp/view.asp?a=2719&q=325532&depNav_GID=1654)

A protozoan parasite was detected in histological sections, especially in the nerves. Ultrastructurally, a parasome was evident; therefore, the parasite was morphologically close to the genus *Paramoeba* Schaudinn 1896 (see Mullen et al. 2005). The lobsters exhibited red discolouration of muscle and hemolymph, pigment deposition in hemocoelomic viscera, coelomic hemolymph and marked nodular swelling of segmental ganglia of ventral nerve cords (Mullen et al. 2004). Sixty percent of the animals examined had mild to moderate hemocytic infiltrates with intralesional protozoa in nerves and ganglia (supraoesophageal and segmental ganglia) (Mullen et al. 2004). Amoebae were also found in the ventral nerve cord, optic and antennal nerves, retina and eyestalks (Mullen et al. 2004).

Different stress factors were suspected to having contributed to the mass mortality. Pesticides used by New York and Connecticut state environmental agencies to control mosquito populations carrying West Nile Virus were suspected to be implicated in causing immunosuppression and stress to the LIS lobsters (Anderson et al. 1999). However, mortalities of lobsters were already reported in fall of 1997 and 1998 (CTDEP 2000), before the use of pesticides in this area. Miller et al. (2005) indicated that concentrations of individual pesticides in the LIS water were too low to cause adult lobster mortality in 1999. Additionally, toxicological analysis failed to detect the presence of the incriminated pesticides, i.e. malathion, methoprene, and resmethrin (Mullen et al. 2004). Tissue samples were also unsuccessfully screened for toxic phytoplankton and bacteria (Mullen et al. 2004).

Temperatures for the LIS region have been increasing since 1998 and hypoxic episodes have occurred regularly (Varekamp et al. 2004). Long Island Sound waters

have remained several degrees warmer than usual for sustained periods (Varekamp et al. 2004). In summer and fall 1999, the bottom temperature was unusually high (23 °C), increasing the respiration rate and disrupting the acid-base status and the serum chemistry in lobsters (Dove et al. 2005). In late August, violent winds mixed the water column and exacerbated the rise of temperature, compromising weakened lobsters (Wilson & Swanson 2005).

Although lobsters in LIS do not usually move extensive distances (less than 10 km a day) (Howell et al. 2005), they can escape hypoxic conditions by migrating to the nearest cooler deeper water. During summer 1999, lobsters presumably concentrated in certain regions of western LIS. Low dissolved oxygen combined with warm temperatures at the water-sediment interface caused the release of sulfites and ammonia from the sediments (Cuomo et al. 2005), enhancing the susceptibility of crowding lobsters to parasitic infection (Draxler et al. 2005). Presently, it is still difficult to determine the respective contribution to the massive lobster mortality of environmental stressors such as extended periods of hypoxia, exposure to sulfites and ammonia, warmer bottom temperatures, and the amphizoic amoeba, *Neoparamoeba pemaquidensis*.

1.2 Complexity of Amphizoic Amoebae

1.2.1 Unstable Classification Scheme

Different species of amoebae have been incriminated in mass mortalities of marine fish and invertebrates. They were subsequently named and identified on the basis of cell size, morphology of locomotion, surface decorations, and host species: *Paramoeba perniciosa* (see Sprague et al. 1969) in blue crab, *Callinectes sapidus*; *Paramoeba invadens* (see Jones 1985) in green sea urchin, *Strongylocentrotus droebachiensis*, *Neoparamoeba pemaquidensis* in American lobster, *Homarus americanus* (see Mullen et al. 2005), and finfish (Kent et al. 1988, Howard & Carson 1993, Dyková et al. 1995, Dyková et al. 2000, Munday et al. 2001). However, molecular sequence data (Mullen et al. 2005) indicate that parasitic *Neoparamoeba* from lobsters, sea urchins and finfish all belong to a single clade, and should probably be considered representatives of a single species even though their respective pathogenesis and morphological features are different.

Traditional classifications of Protozoa, including amoeboid organisms, are mainly based on morphological approaches (see Figure 1. 2 for example on Gymnamoebae). For more than 20 years, classification has undergone many changes due to the better understanding of phylogenetic lineages. Recently, efforts have been made to improve the stability of the classification scheme (Adl et al. 2005). Overall, it is still difficult to refer to a specific taxonomy since the discrepancies among available publications are enormous. Morphological criteria are important but do not provide clear

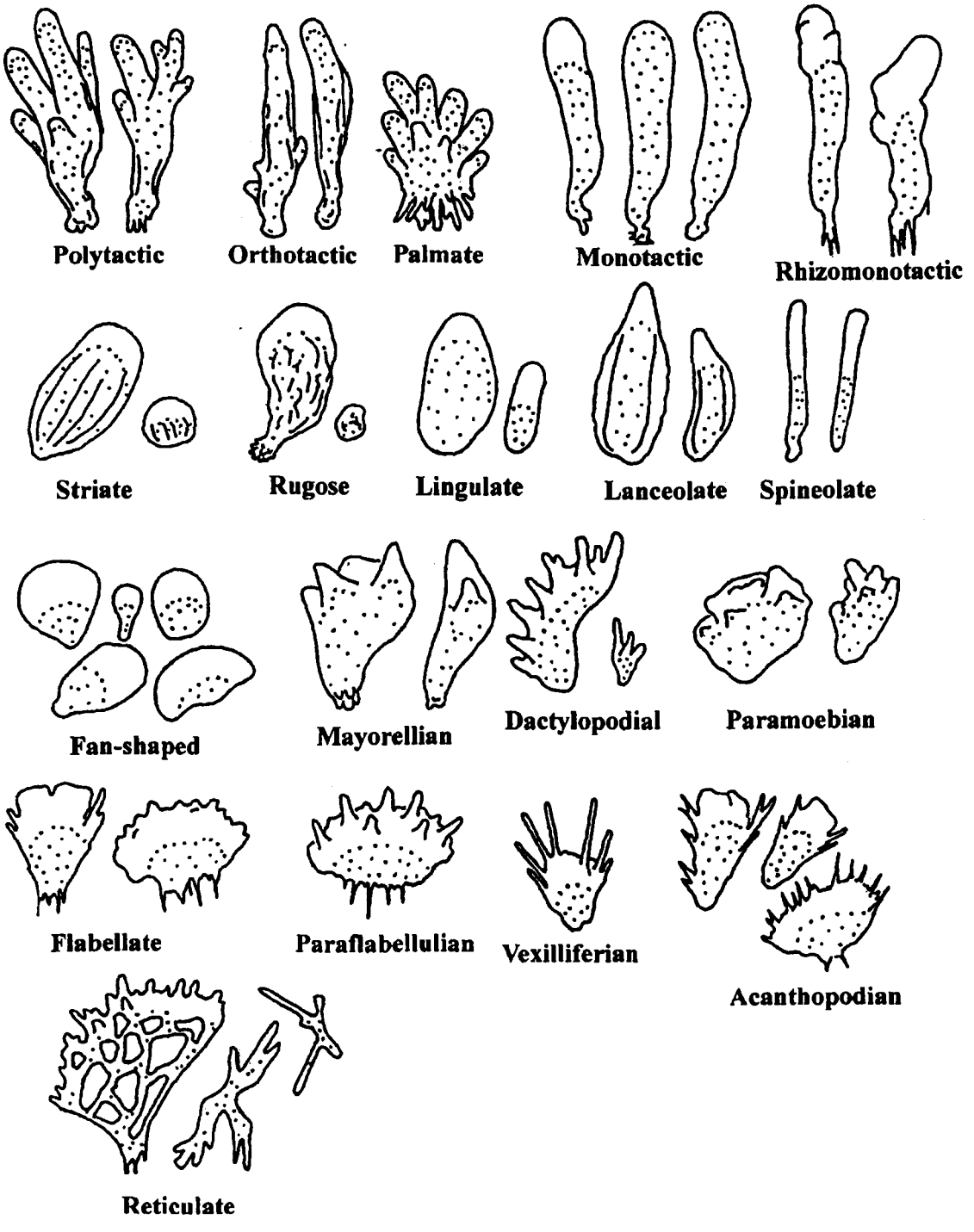

Figure 1.2. Morphotypes of Gymnamoebae (adapted from Smirnov & Goodkov 1999).

indications for a definitive classification of amoebae. New approaches have included descriptions of growth, nutritional characteristics, serologic responses, mitosis, motility, virulence, protein profiles and DNA restriction fragment-length polymorphism analysis (Bracha & Mirelman 1984, Gromov 1985, Ferrante 1991, Schuster 2002, Schuster & Visvesvara 2004a, Wang & Ahearn 2004, Adl et al. 2005, Visvesvara et al. 2005a, Tsutsumi & Shibayama 2006).

Amoebae are quite diverse and range in size from the largest known protist *Pelomyxa*, at several mm, to the tiny amoeba, *Metachaos diminutivum* at 10-20 μm (Bovee 1985). The amoeboid cell body is structurally simple; the plasmalemma may be either naked or possess an external test. Some groups present an internal skeleton: *Difflugia* has a test or shell composed of small pebbles in a protein-based gel (Bobrov & Mazei 2004). Other amoebae such as *Paramoeba eilhardi* exhibit scales (Grell & Benwitz 1966) whereas the surface coat of *Neoparamoeba pemaquidensis* is subdivided into hexagonal glycostyles (Page 1987) and is further characterized by a thick semi-protective mucoprotein layer (glycocalyx) of approximatively 10 nm (Dyková et al. 2000).

The presence of a nucleus-like organelle or parasome within the amoeba constitutes a criterion of classification. It is also described under other names such as "Nebenkörper" (German for "secondary body") or "paranucleus". The parasome ("additional body" in Greek) separates species of *Paramoeba*, *Neoparamoeba* and *Janickina* from all other amoebae. *Neoparamoeba pemaquidensis* contains one or several parasomes that are localized near the nucleus (Figure 1.3).

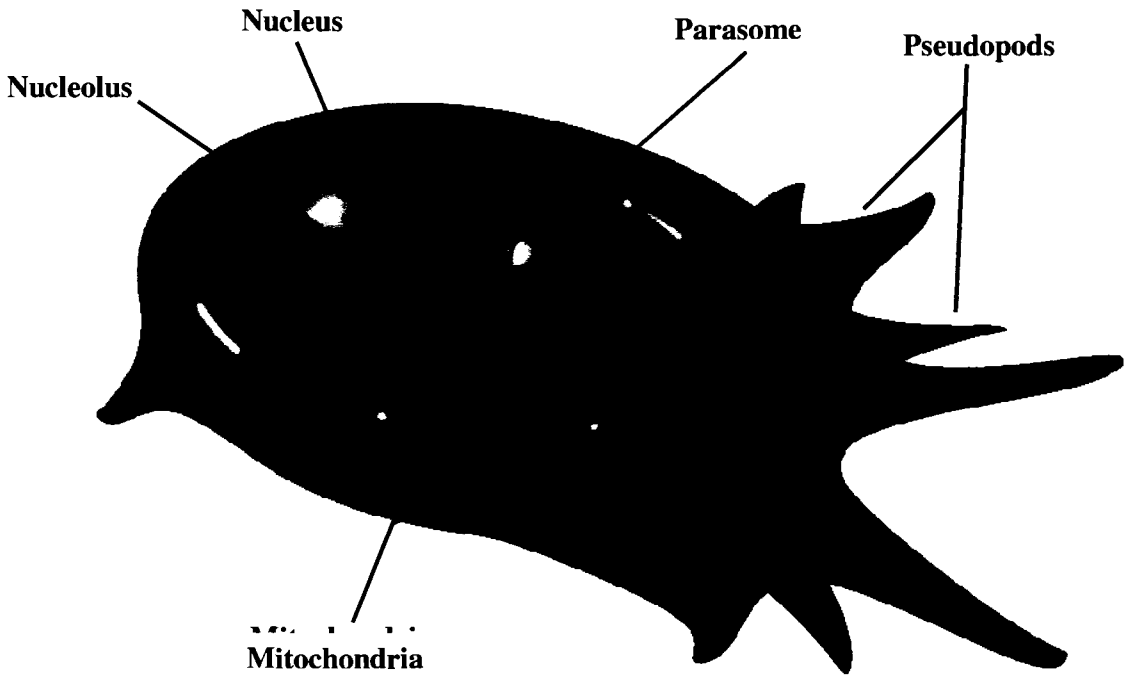

Figure 1.3. Schematical organisation of *Neoparamoeba pemaquidensis*. Adapted from Caraguel and used with permission.

The structure and the reproduction of the parasome have been examined many times (Schaudinn 1896, Janicki 1912, De Faria et al. 1922, Minchin 1922, Janicki 1928, Hollande 1940, Chatton 1953, Grell 1961, Kudo 1966, Grell 1968, Sprague et al. 1969, Grell & Benwitz 1970, Page 1970, Perkins & Castagna 1971). The parasome is approximately the same size as the amoeba nucleus but is more oblong and consists of two peripheral bodies and a central body containing DNA (Grell 1961). The exact origin and biological importance of this structure has proven difficult to elucidate, even with the use of phylogenetic studies based on the 18S ribosomal RNA gene (Dyková et al. 2003, Moreira et al. 2004), which showed that this endosymbiont is closely related to the kinetoplastid, *Ichthyobodo necator*. These endosymbionts have not been isolated and cultured independently; they likely have an intimate and obligate association with their *Neoparamoeba pemaquidensis* host (Hollande, 1980, Caraguel 2006).

Generally, free-living amoebae have two developmental stages: the vegetative trophozoite and the quiescent cyst. Species such as *Naegleria* sp. possess an additional flagellate stage. The trophozoite (amoeboid cell) is the proliferative form in which the plasmalemma lacks a permanent shape. It may be naked or bear a cell coat (glycocalyx). Pseudopodia, which are formed by hyaloplasm and granular endoplasm, are used for both locomotion and feeding. Monopodial amoebae (e.g. Hartmannellidae, Entamoebidae) may move as one mass (e.g. "limax amoeba") as opposed to polypodial amoebae (e.g. Acanthamoebidae), that use more than one advancing point. Other amoebae produce secondary projections, sub-pseudopodia, from one large pseudopodium. Pseudopodia represent a major character used in amoeba classification for identification (Page 1976).

The cysts are also very useful in classification of free-living amoebae (Bowers & Korn 1969, Visvesvara et al. 2005a). The most characteristic features of cysts are shape, appearance of the wall (both inner and outer layers) and presence and morphology of scales. Cysts are generally formed when adverse conditions prevail including varying pH, osmotic pressure, and temperatures. The proliferative form can be restored when favourable conditions arise. Amoebae leave the cyst by rupturing or digesting the wall or through a pre-formed pore, from which a plug is removed (Bovee 1985). In *Neoparamoeba* spp., the presence of a cyst stage has never been observed.

The number of chromosomes depends on the species of amoeba: *Entamoeba histolytica* has 6 chromosomes (Arguello et al. 1992) whereas *Amoeba proteus* has several hundred chromosomes (Gromov 1985). Sexual reproduction is unevenly distributed among protozoa, and has never been observed in lobose amoebae. Species of *Paramoeba*, as in all other naked amoebae, reproduce asexually by binary fission, with mitotic division of the nucleus (Page 1970). The nucleolus and nuclear envelope are no longer visible after prophase. Spindle microtubules extend from discrete poles to the chromosomes. Cytokinesis normally occurs immediately after completion of mitosis. No centrioles or other obvious structures are present at the poles. Division of the parasome also occurs, either simultaneously with the nucleus or before cytokinesis (Page 1970). Mitotic events are usually difficult to observe in amoebae. However, Biron et al. (2001) demonstrated that *Entamoeba invadens* entering mitosis were "helped" by neighbour cells to divide. These cells are called midwives and are likely supplying additional supportive traction. This cooperation displayed by dividing amoebae may be compared to assisted reproduction and suggests one of the earliest forms of intercellular

communication. Interest in amphizoic amoebae has increased recently because of their pathogenicity to humans and animals and their role as reservoirs for amoeba resistant bacteria (ARB) such as *Legionella pneumophila*.

1.2.2 Dual Ecological Roles of Amoebae

1.2.2.1 Regulation of Bacterial Populations by Amoebae

Amoebae are important ecologically because they occupy numerous niches. In soil, amoebae generally colonize plant-soil interfaces since plants allow growth of various bacteria and fungi on which amoebae can feed. Biofilms in soil and water provide ideal conditions for free-living amoebae to proliferate. Many free-living amoebae serve as predators and control bacterial populations (Rodriguez-Zaragoza 1994); other amoebae develop symbiotic relationships with microorganisms (Greub & Raoult 2004).

In trophic relationships, amoebae display pronounced attractions for certain bacterial food sources such as *Klebsiella pneumoniae* and *Escherichia coli* (Weekers et al. 1993). The amoeba *Naegleria* has the ability to discriminate between edible and non-edible bacteria and also can avoid toxin-producing bacteria *in vitro* (Marciano-Cabral & Cline 1987). In the environment, amoebae consume bacteria by phagocytosis or food-cup formation. Engulfed bacteria are then destroyed by pore-forming polypeptides that permeabilize the bacterial cell membrane (Herbst et al. 2002). Ingestion rates may be very high, especially when amoebae divide intensively. For instance, *Entamoeba histolytica* may ingest up to 100 bacteria/ h (Bracha et al. 1982).

The causative agent of amoebic gill disease, *N. pemaquidensis*, is an amoeba that is readily isolated from the marine environment, in particular from tidal marshes, intertidal zones and sediments (Page 1970). Also, the presence of *Neoparamoeba* sp. on biofouled salmon nets and in surrounding waters indicates the environment is a potential reservoir of the agent of AGD (Clark & Nowak 1999, Tan et al. 2002, Douglas-Helders et al. 2003a).

Hall & Voelz (1985) isolated a prokaryotic endosymbiont from an *Acanthamoeba* sp. that was non culturable on artificial media. Jeon & Jeon (2004) showed that bacterial symbionts could suppress the expression of genes in the host amoeba, *Amoeba proteus*. This suggests that amoebae are required for the survival and multiplication of endocytic bacteria. However, the stability of the amoeba-bacteria relationship may depend on environmental conditions: by increasing the incubation temperature of *Acanthamoeba* from 22 °C to 30-37 °C, Birtles et al. (2000) caused the amoebae to be lysed by their bacterial endosymbionts.

1.2.2.2 Dispersion of Bacterial Populations by Amoebae

The importance of amoebae in transmission and dispersion of bacterial pathogens has mostly been evaluated with respect to human disease (Barker & Brown 1994, Greub & Raoult 2004). Some bacteria survive uptake, and use amoebae as hosts for avoiding adverse environmental conditions. For instance, Rowbotham (1986) observed that *Acanthamoeba* containing *Legionella* (causative agent of legionnaire's disease) can encyst, thus protecting the bacteria against high temperature or biocides. These bacteria have become resistant to the amoebae that engulf them by defence

mechanisms such as toxins or outer-membrane structures (Weekers et al. 1993). ARB are bacteria that have evolved mechanisms to avoid the normal digestion processes of their free-living amoeba hosts. These ARB affect humans or animals and include *Listeria monocytogenes* (see Ly & Muller 1990), *Pasteurella multocida* (see Hundt & Ruffolo 2005), *Vibrio cholerae* (see Thom et al. 1992), *Mycobacterium avium* (see Cirillo et al. 1997), *Campylobacter jejuni* (see Axelsson-Olson et al. 2005), and *Legionella pneumoniae* (see Shadrach et al. 2005).

Beyond ensuring protection to bacteria, the amoebae are important in propagation, distribution in the environment, and transmission of bacteria to suceptible hosts. Excystation (i.e. the release of the cyst internal content) of trophozoites harbouring bacteria silmutaneously leads to the release of these bacteria in the environment (Marciano-Cabral 2004). Rowbotham (1986) suggested humans become infected with *Legionella pneumophila* by inhaling amoebae containing endocytic bacteria. Finally, free-living amoebae can serve to pre-adapt bacteria to invasion and intracellular survival within other eukaryotic cells, such as mammalian macrophages (Cirillo et al. 1999).

Although amoebae can enhance bacterial cytopathogenicity by preventing detection of the bacteria while in the amoeba (Fritsche et al. 1998), it may be difficult to determine the respective contribution of the bacteria and amoebae to virulence and pathogenicity. Most bacteria-amoeba associations have been observed under laboratory conditions and their occurrence and stability in nature are unknown.

1.2.3 Challenging Pathogen-Host-Environment Relationship of Amphizoic Amoebae

All protozoan phyla contain parasitic genera; some are exclusively parasitic, including Apicomplexa, Myxozoa and Microspora (Baker 1982). The host-parasite relationship of amoebae appears less clearly defined. Although amoebiasis caused by the obligate parasite *Entamoeba histolytica* is one of the most devastating diseases in tropical regions, most amoebae were considered saprophytic and non pathogenic until Culbertson et al. (1958) first established the pathogenic potential and characteristics of *Acanthamoeba* in the late 1950s. The discovery that free-living amoebae may produce fatal infections caused renewed interest in these organisms.

The importance of a disease relies upon the interaction of factors derived from the pathogen, host and environment, which are often depicted as three interlocking circles with disease occurring at their intersection. As many variables may be unknown, it is often difficult to evaluate these complex interactions. However, identification of risk factors is required before preventive measures can be developed and disease managed efficiently (Thrusfield 1995). This requires knowledge of host and pathogen characteristics, and environmental factors that promote disease.

1.2.3.1 The parasites

Factors associated with the pathogen include route of infection (the route of entry of the pathogen to the host: oral, dermal or branchial in fish), duration of exposure (length of exposures, number of times the individual is exposed, and the time between exposures), infective dose (amount of disease-causing organism that is required to infect

an individual), and pathogenicity of a given parasite. The first three factors depend highly on the species of amoeba. In this thesis, pathogenicity is defined as the potential ability to produce disease (Steinhaus & Martignoni 1970). Virulence is the degree of pathogenicity of a microorganism as indicated by the severity of the disease produced (Steinhaus & Martignoni 1970). Infectivity is a measure of the ability of a disease agent to establish itself in the host (Thomas & Elkinton 2004). In AGD, the main reservoir of *N. pemaquidensis* is infected fish (Zilberg et al. 2001). A single infected fish may carry a few million amoebae, which can survive on gills up to 3 days after the fish dies (Dyková & Novoa 2001). The minimal infective dose is similar to the concentration of amoebae found naturally in the sea water column, i.e. 10-50 cells / L (Morrison et al. 2004).

The mechanisms by which amphizoic amoebae cause cell damage are still poorly understood. To date, studies of amphizoic amoebae have focused on those affecting humans, especially *Acanthamoeba* spp. and *Naegleria* spp. (see Ferrante 1991, Niederkorn et al. 1999, Shin et al. 2000, Khan 2001, Mattana et al. 2001, Kinnear 2003). *Acanthamoeba* invasion of the eye involves the elaboration of proteases that facilitate cytolysis of the corneal epithelium, invasion of the extracellular matrix, and dissociation of the corneal stromal matrix (Niederkorn et al. 1999). Additionally, *Acanthamoeba* trophozoites have exhibited mechanical cytopathic effects *in vitro* as they roamed over the surface of cultured keratocytes, passing between cells and squeezing into the gaps. Consequently, keratocytes detached from the substratum (Kinnear 2003). Shin et al. (2000) showed that some rat microglial cells co-cultured with pathogenic *Acanthamoeba culbertsoni* undergo apoptosis. Mattana et al. (2001) demonstrated cytolytic action of

Acanthamoeba castellanii on human epithelial Wish cells (human amnion derived cells) that culminated in apoptosis. A combination of physical and chemical factors is likely responsible for the damage to affected cells.

Unlike mammalian amoebic diseases, there is no evidence of cytolytic activity *in vivo* against host tissue when viewed with light, TEM or SEM preparations. Histological sections of AGD lesions show intact proliferating cells in close association with amoebae and extensive inflammation. Additionally, Atlantic salmon exposed to an amoebic lysate and possibly extracellular products did not develop AGD lesions (Adams & Nowak 2004a). However, *Neoparamoeba pemaquidensis* is cytolytic towards cultured gill epithelial cells *in vitro* (Butler & Nowak 2004, Lee et al. 2006).

1.2.3.2 The Hosts

Generally, host factors of primary importance are species, sex, age, immune status, and population size. Amphizoic amoebae infect a wide range of invertebrate and vertebrate hosts. In humans, central nervous system diseases caused by amphizoic amoebae include Primary Amoebic Meningoencephalitis (PAM) due to *Naegleria fowleri* and Granulomatous Amoebic Encephalitis (GAE) due to *Acanthamoeba* spp. and *Balamuthia mandrillaris* (see Visvesvara et al. 1993, Marciano-Cabral et al. 2000, Cabanes et al. 2001, Marciano-Cabral & Cabral 2003). Some *Acanthamoeba* produce keratitis in soft contact lens wearers, which can cause visual impairment (Moore et al. 1987).

Acanthamoeba spp. also cause central nervous system infections in dogs (Brofman et al. 2003, Dubey et al. 2005) whereas *N. fowleri* have been isolated from the

brain of cows that died of PAM (Visvesvara et al. 2005b). Sesma & Ramos (1989) reported infections of *Naegleria* and *Acanthamoeba* in lizards on the Canary Islands (Spain). Several aquatic animals may be naturally resistant to amoebae (John & Smith 1997). For example, serum of the alligator, *Alligator mississippiensis,* exhibits broad-spectrum amoebicidal activity (Merchant et al. 2004).

Finfish and shellfish are susceptible to opportunistic amoebae. In most amoebic diseases described in aquatic organisms, detailed identification of the amoebae was not possible due to challenges of isolating and cultivating the parasites (Nash et al. 1988, Voelker et al. 1977, Daoust & Ferguson 1985, Speare 1999, Smith et al. 2002). Cases of systemic amoebiasis were reported in finfish including the cultured European catfish, *Silurus glanis* (see Nash et al. 1988) and goldfish, *Carassius auratus* (see Voelker et al. 1977). This latter infection was characterized by granulomatous organ lesions. Sporadic cases of branchial amoebiasis have also been diagnosed. Firstly, Daoust & Ferguson (1985) described a form of rainbow trout gill disease. Then, Speare (1999) described a similar nodular gill disease in Arctic char, *Salvelinus alpinus*. Finally, Smith et al. (2002) reported an amoebic gill infestation in the pallid sturgeon, *Scarphirhynchus albus*.

Gill-infecting amoebae such as *Neoparamoeba*, *Cochliopodium*, and *Thecamoeba* spp. are the most prevalent amoebic infestations in fish (Page 1988). Generally, various epiphytic (living within or upon another without causing harm) species of amoebae may be found on the gills. For instance, amoebae belonging to the genera *Platyamoeba*, *Vannella*, and *Flabellula* were found with *Neoparamoeba*, the supposed agent of amoebic gill disease, on infected turbot, *Scophtalmus maximus* (see

Dyková et al. 1999). *Neoparamoeba pemaquidensis*, additional to causing severe chronic amoebic gill disease in maricultured fish, especially salmonids (Kent et al. 1988), can infect invertebrates including green sea urchins, *Strongylocentrotus droebachiensis* (see Jones 1985, Jones & Scheibling 1985, Scheibling & Hennigar 1997), and American lobsters, *Homarus americanus* (see Mullen et al. 2004). Surprisingly, *N. pemaquidensis* has systemic distribution and displays pronounced neurotropism in invertebrates whereas in finfish, it is a gill ectoparasite.

In terrestrial hosts, amphizoic amoebae cause sporadic diseases and are transmitted from the contaminated environment to the host. In contrast, the marine amoeba *N. pemaquidensis* is directly transmissible from host to host and may devastate an entire population of marine vertebrates or invertebrates (Jones 1985, Kent et al. 1988, Mullen et al. 2004). Experimental infections of AGD in finfish can only be established by cohabitation of naïve with infected individuals or by exposure (water-borne-route) to freshly isolated parasites (Zilberg et al. 2001).

The importance of the host health status in development of AGD is not well understood. Some studies suggested that the amoebae may only occur on gills with pre-existing lesions induced by other parasites or by chemical or mechanical injuries (Kent et al. 1988, Zilberg & Munday 2000, Douglas-Helders et al. 2003a, Bermingham & Mulcahy 2006). Several studies showed opposite results, i.e. pre-existing gill lesions in Atlantic salmon did not predispose gill epithelia to colonization by *Neoparamoeba* sp. (Zilberg & Munday 2000, Adams & Nowak 2004b). Fish do not acquire resistance against the amoeba (Gross et al. 2004). Although salmonids experimentally develop local and systemic immune responses when exposed to *N. pemaquidensis*, they are not

protected from re-infection (Akhlaghi et al. 1996, Zilberg & Munday 2001, Bridle et al. 2006).

1.2.3.3 The Environment

The environment is an integral component of the pathogen-host-environment relationship affecting development of disease (Hedrick 1998). Pathogens need adequate conditions to survive, enter the host and proliferate. Environmental factors can be classified as physical or biological. Understanding the role that environmental factors play in disease occurrence and propagation is necessary in selecting fish farming sites and for avoiding areas affected by human activities. This also allows prediction of disease events (e.g. AGD outbreaks occur in the fall when water temperature peaks).

Globally, the exact factors triggering the invasion and development of amphizoic amoebae in the host are not well understood. However, elevated temperatures are strongly implicated in the proliferation and pathogenicity of the amoebae. In humans, putative pathogenic amoebae such as *Naegleria fowleri* appear to multiply more rapidly during warmer seasons (John 1993).

In fish, disease development is affected by several water quality parameters, including temperature, salinity, water hardness, pH, ammonia, nitrite, nitrate, suspended solids, and dissolved gas concentrations (Snieszko 1973). The effects may be on the pathogen, host immune status or both. Generally, as water temperature increases, the rate of parasite development increases. For instance, paramoebiasis outbreaks in invertebrates and vertebrates usually occur at high water temperatures (> 15 °C) (Kent et al. 1988, Scheibling & Hennigar 1997, Douglas-Helders et al. 2001b, Mullen et al. 2005).

Severity and duration of an outbreak of AGD is primarily a function of high salinity (32 ‰) and high water temperature (12-20 °C) (Clark & Nowak 1999). In the American lobster, stress factors, such as hypoxia and chemical pollution in the LIS die off, may have compromised the host immune system; however it is difficult to assess contributions to the pathogenicity of the amoeba (Mullen et al. 2004).

1.3 Challenges of Diagnosis and Treatment of Amoebic Diseases

1.3.1 Diagnosis

Etiological diagnosis is of primary importance in any disease issue. In humans and terrestrial animals, systemic infections caused by amphizoic amoebae are difficult to diagnose because they have no pathognomonic signs, and they are rare and underreported. Diagnosis is often made post-mortem (Voelker et al. 1977, Schuster & Visvesvara 2004b). In humans, infections caused by the amoebae *Acanthamoeba, Naegleria* and *Balamuthia* are either detected in tissue sections with hematoxylin-eosin or by indirect immunofluorescence staining using rabbit anti-amoeba serum. Serological screening of patients with suspect encephalitis can detect anti-amoeba antibodies during *Acanthamoeba* and *Balamuthia* infections (Visvesvara et al. 1993). However, species identification is difficult because reliable diagnostic tests based on species specific antibodies are not available. Epidemiological analysis may be helpful. For instance, PAM occurrence (caused by *Naegleria*) is strongly associated with water activity such as swimming (John 1993). By contrast, a localized amoebic infection such as human acanthamoebic keratitis is readily detected because of its effects on vision. Cysts may be

identified in corneal scrapings or may be cultured on non-nutrient agar with bacteria, for identification (Schuster 2002).

The severity of AGD in Atlantic salmon is evaluated by the presence, size and number of white mucoid patches on the gills during gross examination (Alexander 1991). However, this presumptive on farm diagnosis is only reliable in areas with high prevalence and intensity of AGD (Adams et al. 2004). Without this epidemiological correlation, large discrepancies may be reported between gross gill examination in the field and histological findings (Clark & Nowak 1999). The "characteristic" white mucoid patches are not always visible in AGD-affected Atlantic salmon (Palmer et al. 1997), and using this criterion for the diagnosis of AGD is not applicable to other finfish species such as rainbow trout in which the gill pathology differs from that of Atlantic salmon (Munday et al. 2001). Using gross observations only can be further misleading as *Neoparamoeba* sp. was isolated from turbot without gross evidence of disease (Dyková & Novoa 2001). To overcome this deficiency, Zilberg et al. (1999) recommended the use of Quick Dip® (Fronine Pty Ltd, Riverstone, NSW, Australia) stained gill smears instead of gross gill checks as a fast method to confirm AGD outbreaks on affected farms.

In diagnostic laboratories, investigations of AGD outbreaks generally rely upon two diagnostic methods, i.e. gill histopathology and indirect fluorescent antibody test (IFAT) of gill smears or sections (Howard & Carson 1993). The IFAT is accepted as the gold standard for verification of AGD associated with *Neoparamoeba* sp. (see Palmer et al. 1997, Douglas-Helders et al. 2001a) and can be used for the detection of amoebae in water samples (Douglas-Helders et al. 2003a). Full agreement between histological

diagnosis and IFAT results has been demonstrated, confirming that they are reliable diagnostic methods for AGD (Clark & Nowak 1999). However, these methods have the disadvantage of requiring lethal sampling for diagnosis and are not suitable for processing very large numbers of samples because of slide reading fatigue. Douglas-Helders et al. (2001b) developed a dot blot assay using non-lethal gill mucus samples to detect the presence of *Neoparamoeba* spp. The immuno-dot-blot is highly correlated with IFAT and is convenient for screening large numbers of samples.

A limitation to the use of IFAT was recently discovered; the antiserum cross-reacted with the closely related and non pathogenic species, *Neoparamoeba aestuarina* and *Pseudoparamoeba pagei* Page 1979 (see Douglas-Helders et al. 2001b). The IFAT may be further compromised by the discovery of a new species isolated from turbot and Atlantic salmon, *Neoparamoeba branchiphila* (see Dyková et al. 2005). The detection of *N. pemaquidensis* by the IFAT is unknown or has not been published.

The above commentary illustrates that detection of pathogens in human and veterinary medicine is becoming more dependent upon molecular biology techniques (Leong 1995). Techniques such as Polymerase Chain Reaction (PCR) are very sensitive and accurate, and have been useful in identifying individual genera and species of pathogens that affect economically important aquaculture species. Molecular characterisation of *Neoparamoeba pemaquidensis* isolates has been developed for detection of the causative agent of AGD and is suitable for use with fish gills, sea water and biofouling samples (Elliott et al. 2001, Fiala & Dyková 2003, Caraguel 2006). The practical relevance of molecular identification of the different genera is that it allows for

formulating decisions with respect to the selection of the appropriate chemotherapy and prognosis, as there is no universal cure for amoebic infections.

1.3.2 Treatments

In humans, drugs that have been used to treat systemic amoebic diseases caused by *Acanthamoeba* spp. include ketoconazole, sulfadiazine, and pentamidine isethionate (Schuster & Visvesvara 2004b). The drugs of choice to treat acanthamoebic keratitis are two cationic antiseptics, chlorhexidine gluconate and polyhexamethylene biguanide (Schuster & Visvesvara 2004b). However, reinfection due to resistant amoeba cysts may lead to to an occular condition that necessites repeated corneal transplants. The drug of choice to eradicate *Naegleria fowleri* is the antifungal polyene antibiotic Amphotericin B (alters cell membrane permeability) (Schuster & Visvesvara 2004b). *Balamuthia mandrillaris* is sensitive to fluconazole, azithromycin (50S ribosomal subunit protein synthesis inhibitor) and flucytosine (Schuster & Visvesvara 2004b).

In mammals and birds, there are relatively few amoebae of importance and these are rarely associated with clinical disease. Consequently, unlike in human medicine, specific amoebicidal drug screening and testing has been limited in veterinary medicine. Currently available drugs (nitroimidazoles, pentavalent antimonials, arsenicals...) are grouped under the general denomination "anti-protozoal" rather than "amoebicidal" because they primarily target common parasitic flagellates such as *Trichomonas*, *Giardia*, and *Leishmania* found in domestic animals (Lindsay & Blagburn 1995).

In fish farming, few drugs are permitted and their use is strongly restricted due to the risks of environmental pollution, toxicity to fish, unknown withdrawal times, and

antimicrobial resistance (Burka et al. 1997). Sea cages offer little control over the environment, thus making treatment difficult. However, as many factors affecting transmission are unknown or difficult to control, drug testing must therefore, be considered as an important option for the continued sustainability of the aquaculture industry. Amoebic gill disease may be only partially controlled by the use of curative freshwater bath treatment and other appropriate husbandry practices such as reducing stocking densities (Findlay et al. 1995, Parsons et al. 2001). Research efforts have mainly focused on risk factor identification for preventing and containing disease. Very little research exists regarding the *in vitro* susceptibility of amoebae to drugs (Powell et al. 2003). Consequently, effective drugs against *N. pemaquidensis* are still unavailable. In wild caught invertebrates, which may be held for extended periods before being marketed, there is no cure for paramoebiasis and no research on this topic has been conducted. Developing and testing of drugs will require access to standardized models of paramoebiasis and culture methods.

Currently, the main model used in AGD research is the *in vivo* model, i.e. Atlantic salmon (Munday et al. 2001). The use of *N. pemaquidensis* cultured on artificial media has been abandoned because it presents too many disadvantages, including loss of pathogenicity after one month on artificial media (Jellett & Scheibling 1988, Kent et al. 1988, Morrison et al. 2005). Although *in vitro* culture using fish derived cell lines as a feeder monolayer has been developed recently (Butler & Nowak 2004, Lee et al. 2006), their potential and limits are unexplored.

1.4 Research Objectives and Specific Aims

Lee et al. (2006) showed that *Neoparamoeba pemaquidensis* rapidly grows on a rainbow trout gill cell line and is cytopathogenic toward the monolayer. The overall research objectives of this thesis were:

1. to compare this cell culture method with the conventional culture methods of *N. pemaquidensis*;

2. to further investigate the characteristics of *N. pemaquidensis* obtained by co-culture with rainbow trout gill cells, especially the potential recovery of pathogenicity; and

3. to test the reliability of this newly established cell-culture system in providing a consistent supply of amoebae for experiments.

The specific aims of this thesis were:

1. Review of the culture methods available in paramoebiasis research (Chapter II);

2. Attempt to induce successful water-borne transmission of Amoebic Gill Disease in *Oncorhynchus mykiss* and *Salmo salar* by exposure to *Neoparamoeba pemaquidensis* harvested from rainbow trout gill cell cultures (Chapter III); and

3. Development of a reproducible and rapid viability assay using *Neoparamoeba pemaquidensis* recovered from rainbow trout gill cell cultures (Chapter IV).

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II. CULTURE METHODS IN PARAMOEBIASIS RESEARCH

In vitro isolation and cultivation of numerous pathogenic and opportunistic free-living amoebae affecting humans, such as *Naegleria* and *Acanthamoeba* spp., have been successively performed and furthered the understanding of the basic biology of amoebae. These studies have facilitated the development of mechanisms of prevention, control, eradication and containment (Chang 1971, Schuster 2002). In contrast, the inability to obtain axenically large numbers of parasites appears as the major limitation to many areas of paramoebiasis research. Conventional isolation and culture techniques of amphizoic amoebae affecting aquatic organisms are not reliable and knowledge is still lacking despite recent advances in isolation and maintenance (see Dyková & Lom 2004).

Neoparamoeba research has primarily been restricted to analysis of outbreaks, mainly because the parasite is difficult to isolate and culture on artificial media. Considerable effort has focused on developing and refining an *in vivo* model of paramoebiasis in finfish (Zilberg et al. 2001, Morrison et al. 2004), which is the primary source of parasites in most published experimental studies.

Current methods of maintenance and culture of *N. pemaquidensis* have been evaluated at the AVC Lobster Science Centre (AVCLSC). This chapter describes their respective advantages and limitations.

2.1 Culture of *Neoparamoeba pemaquidensis* on Artificial Media

2.1.1 General Methodology of Isolation and Culture

Screening for the presence of *N. pemaquidensis* in tissues of aquatic organisms or from environmental samples has been based on culture of amoebae on seawater-based agar plates. Attempts at establishing *N. pemaquidensis* cultures directly from gill tissue in liquid media have failed (Dyková et al. 2000). The practice of excising pieces of gill tissues from finfish and placing them on non-nutrient agar such as MY75S has become the general approach to AGD outbreaks (Kent et al. 1988, Page 1988, Dyková et al. 1998, Dyková & Lom 2004), although our laboratory (AVCLSC) has encountered difficulties with this approach. Successful isolation of *N. pemaquidensis* from AGD-infected gill tissue of Atlantic salmon occurred in sea water and liquid media. However, this method gave unreliable results.

During the LIS lobster die-off in 1999, attempts to isolate amoebae from ventral nerve cords and hemolymph samples were performed on MY75S agar and rye grass Cerophyll-sea water agar (CSA) (Mullen et al. 2004). In green sea urchins, amoebae and their associated bacterial populations were successfully isolated from the radial nerve by culture on semi-solid, non nutrient artificial sea water (ASW) agar (0.6 %). Subculturing was performed on solid ASW agar (1.2 %) seeded with the bacterium *Pseudomonas nautica* (Scheibling & Stephenson 1984).

Establishing cultures on transparent agar is essential for observing and monitoring amoeba growth with inverted light microscopy (Figure 2.0). Close scrutiny of cultures is required when bacterial contamination of the gills is heavy, which tends to overwhelm the amoebae. When bacterial contamination occurs, the amount of malt

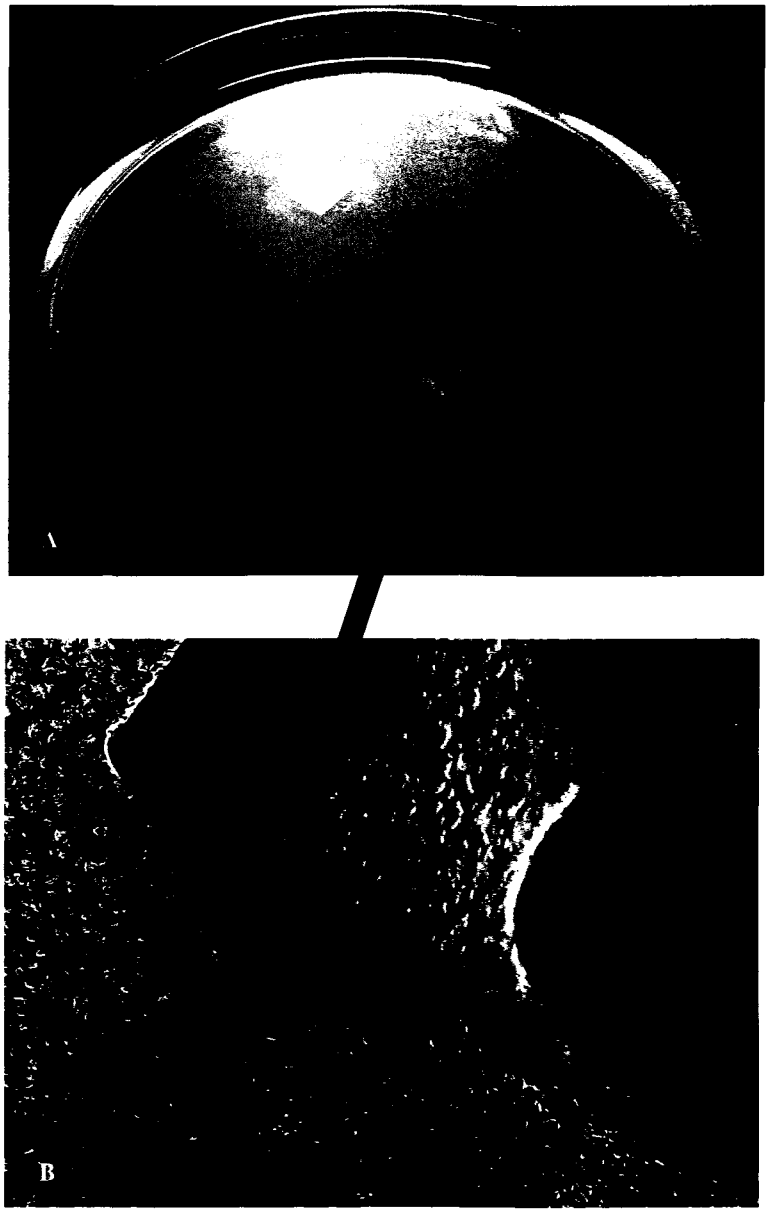

Figure 2.0. Culture of *Neoparamoeba pemaquidensis* CCAP 1560/ 4 on MY75S agar medium. (**A**) *Neoparamoeba* agar plate culture, dense growing zone around the cube delimited by the red circle. (**B**) Healthy *Neoparamoeba* agar plate culture examined with phase contrast Nikon Diaphot-TMD inverted microscope, X 250.

and yeast must be reduced, or alternatively a mixture of antibiotics and antimycotics can be used to reduce the load of micro-organisms present in the tissue: the mixture used by Butler & Nowak (2004) gave satisfying results in our laboratory. The presence of several species of amoebae within decomposing gill tissue may be detected after one month at 20 °C. Some species, such as *Paraflabellula* sp. may outcompete *Neoparamoeba* sp. as they grow faster. *Paraflabellula* sp. was always present in our attempts to isolate *N. pemaquidensis*.

The presence of a parasome in *N. pemaquidensis* is a consistent and helpful diagnostic feature. Our laboratory relies upon this method routinely. Amoebae containing parasomes can be used to select and establish clonal cultures. Clonal subculturing of selected amoebae is time-consuming; individual cells are cut out with a cube of agar and transferred face down onto fresh agar. Similar to what Dyková et al. (2000) found, obtaining a pure culture of *Neoparamoeba* from fish tissues may take more than 2 months in our laboratory. The confirmation of trophozoite identity may be performed by PCR (Elliott et al. 2001, Tan et al. 2002, Caraguel 2006), TEM (Roubal et al. 1989, Dyková et al. 2000), or IFAT (Tan et al. 2002); we currently use PCR.

Trained observers can recognize growth characteristics of a given isolate *in vitro*. Certain unique features of cultured amoebae such as pigment production can be used as an aid in the identification of the amoebae: in our laboratory, ATCC 30735 and AVCLSC-001 cultures appear yellowish and greasy; CCAP 1560/ 4 and CCAP 1460/ 5 form whitish opaque delimited cultures around the cube of agar. The AVCLSC-001 culture progressively destroys the agar layer. Table I lists our isolates of *N. pemaquidensis* and their culture requirements on artificial media.

Table I. *Neoparamoeba pemaquidensis* isolates cultured on artificial media

Label	Origin	Location	Media	Feeder bacteria	Incubation Temperature
AVCLSC-001	Atlantic salmon *Salmo salar*	WA, USA	ATCC 994	*Klebsiella pneumoniae*	20 °C
ATCC 30735 Clone CH-27	Environment	VA, USA	ATCC 994	*Klebsiella pneumoniae*	20 °C
ATCC 50172 Clone A	Coho salmon *Oncorhynchus kisutch*	WA, USA	ATCC 994	*Klebsiella pneumoniae*	20 °C
UA1 Bigelow Laboratory	Green sea urchin *Strongylocentrotus droebachiensis*	ME, USA	L1	*Enterobacter aerogenes*	16 °C
UA2 Bigelow Laboratory	Green sea urchin *Strongylocentrotus droebachiensis*	ME, USA	L1	*Enterobacter aerogenes*	16 °C
CCAP 1560/ 4	Environment	Wales, UK	MY75S	No added bacteria*	20 °C
CCAP 1560/ 5	Environment	Wales, UK	MY75S	No added bacteria*	20 °C

* CCAP 1560/ 4 and 1560/ 5 are co-cultured with a resident unidentified bacterial population

Since amoebae isolated from tissues are associated with bacteria, there is no need to add bacteria to agar plates in the early period of subculturing. However, unlike most *Naegleria* and *Acanthamoeba* spp. (see Schuster 2002), *N. pemaquidensis* isolates do not readily adapt to bacteria-free medium. Overall, protocols for feeding *N. pemaquidensis* vary with the isolate (Table I). Several species of bacteria have been used as a food source for *N. pemaquidensis*, including *Escherichia coli* (see Sprague et al. 1969), *Klebsiella pneumoniae* (see Peglar et al. 2003), *Enterobacter aerogenes* (see Sprague et al. 1969), and *Pseudomonas* spp. (see Scheibling & Stephenson 1984, Akhlaghi et al. 1996, Dyková et al. 2000). We observed that amoeba cultures fed with old subcultured bacterial colonies may grow slower and die. Also, regularly thawing stock bacterial cultures ensures better success in cultivation of *N. pemaquidensis*. Certain isolates of *Neoparamoeba* such as CCAP 1560/ 4 and 1560/ 5 have their own resident population of bacteria and do not need additional food supplies.

Subculturing schedules may vary with the isolate, temperature, humidity, and bacterial growth. Generally, cultures being used in experiments require more frequent transfers (days rather than weeks) than stock cultures. We observed that some isolates such as ATCC 30735 may tolerate long intervals (1 month) at which cultures have to be transferred. Others, such as ATCC 50172 are less tolerant and may die within one week. Despite regular transfers, cultures of certain isolates such as the environmental isolate CCAP 1560/ 5 tend to abruptly decline for unknown reasons.

2.1.2 Limitations of *in vitro* Culture on Agar Media

Continuous culture on artificial media is currently the most efficient way to maintain the collection of available *N. pemaquidensis* isolates and to produce pure amoebic cultures at low cost. Ideally, cryopreservation of amoeba isolates would be an alternative to regular subculturing and as a back up in case of culture loss. This is commonly used in several species of free-living amoebae. John (1993) reported that viability of cryopreserved *Naegleria fowleri* was 64 % at 1 month declining to 38 % after 6 months. Similar to what Dyková et al. (2000) reported, we observe that cryopreservation methods of *N. pemaquidensis* using clonal cultures give highly variable outcomes and are not yet a reliable method for preserving cultures.

2.1.2.1 Culture Variability

Culture on artificial media has several disadvantages that limit the use of cultured *N. pemaquidensis* experimentally. First, currently employed methods of isolation and culture maintenance are not successful with all isolates. Although growth of several *Paramoeba*-like organisms in monoxenic cultures on agar plates has been successful (Martin 1985, Jellett & Scheibling 1988), attempts to maintain *Paramoeba perniciosa* from blue crabs in artificial culture as recommended by Page (1970) failed. All original cultures were lost and therefore no longer exist in any culture collection. The longest period of survival, i.e. 2 weeks, was obtained with 10 % foetal bovine serum agar mixed with sterile sea water and held at 18 °C (Sprague et al. 1969). Also, attempts at isolation of amoebae on artificial media from infected lobsters after the Long

Island Sound die off failed (Mullen et al. 2004). Consequently, amoeba isolates from blue crabs and American lobsters are not available.

Secondly, yields of amoebae are generally low and incubation time is long. Martin (1985) reported that the maximal yield of ATCC 30735 cultured in optimal conditions was obtained after 18 days and was as low as 10^4 amoebae/ mL. Jellett & Scheibling (1988) reported a yield of 3.7×10^4 cells/ mL after 29 days of culture. For a given isolate, incubation time may vary throughout the year for unknown reasons. For instance, in our laboratory, CCAP 1560/ 4 is routinely harvested after 10 to 20 days in culture, i.e. when *N. pemaquidensis* forms a 1.5 cm wide belt around the agar cube. Unfortunately, the cultures with the highest yield are generally too senescent to be used in experiments. It is therefore, necessary to prepare a very large number of agar cultures to produce sufficient numbers of young and healthy amoebae. To increase yields, we transferred several agar cubes to a fresh plate. Instead of cube transfer, we also prepared a suspension of amoebae from an agar culture and streaked 1 mL onto a fresh plate with a glass cell spreader. The amoebae readily colonize the whole surface of the plate instead of slowly spreading onto the agar from the cube. However, we observe that young cultures of amoebae are more difficult to detach from agar than older cultures. Consequently, the cost of production and the time necessary to harvest amoebae are increased.

Finally, the continuous propagation of *N. pemaquidensis* on artificial media is highly dependent upon the presence of bacteria. The nature, load and type of bacteria may greatly interfere with the outcome of an experimental study by influencing the growth and behaviour of the amoebae.

2.1.2.2 Differences Between Tissue-Associated and Cultured *Neoparamoeba pemaquidensis*

Conceivably, differences in morphology and culture appearance observed in *N. pemaquidensis* isolates could reflect different strains of a single species living in various habitats and hosts. Roubal et al. (1989) compared the morphology between cultured and gill-attached *N. pemaquidensis* isolated from Atlantic salmon. The cultured amoebae were 13.7 x 19.7μm (n = 20) in average in sea water while the gill-attached amoebae had a body size of 32.9 x 38.1 μm (n = 20), and possessed numerous dense vesicles in the cytoplasm, indicating higher metabolic activity (Roubal et al. 1989). The presence of more than one parasome is generally observed in trophozoites freshly isolated from gill tissue. We observed up to four parasomes in amoebae freshly isolated from salmon gills. Dyková et al. (2000) visualized up to six parasomes in one amoeba and noted that the number of trophozoites with fully developed parasomes tended to decrease in numbers when the culture became old, suggesting a metabolic decline in *N. pemaquidensis.* As clonal cultures aged, there was also a concomitant change in the morphology of the trophozoites which became smaller and elongated (Dyková et al. 2000).

A major challenge associated with culture of *N. pemaquidensis* is the rapid loss of virulence while being maintained on artificial media. Naive coho salmon did not acquire AGD when exposed to cultured amoebae which had been passed 12 to 15 times on artificial medium (Kent et al. 1988). Morrison et al. (2005) showed that trophozoites cultured for 4 weeks did not elicit AGD in Atlantic salmon. In green sea urchins, *in vitro* culture of *N. pemaquidensis* was associated with loss of virulence after 15 to 58 weeks,

depending on the type of bacterial food source (polyxenic versus monoxenic bacterial culture) (Jellett & Scheibling 1988).

Akhlagi et al. (1996) suggested that the most probable reason for lack of immunity when Atlantic salmon are exposed to cultured *Neoparamoeba* sp. is the absence of virulence factors. Villavedra et al. (2005) confirmed that cultured and freshly isolated parasites from infected gills strongly express different antigen profiles. Overall, as the original isolate of *N. pemaquidensis* is cultured on artificial medium, drastic changes occur in morphology, antigen profile, and pathogenicity, which may confound the interpretation of experimental results.

2.2 Maintenance and Propagation of *Neoparamoeba pemaquidensis in vivo*

The limitations of *in vitro* systems may be overcome by developing *in vivo* systems. However, the development and use of an animal model adds another layer of complexity with many interacting factors. A relatively extensive review on animal models used for the study of different amoebic diseases of humans has been published (Tsutsumi & Shibayama 2006).

In vivo models are essential to test the pathogenic potential, or to maintain or restore the virulence of a particular isolate of amoeba. Axenically grown *Entamoeba histolytica* gradually lose their virulence after prolonged cultivation but can regain virulence when trophozoites are passaged *in vivo*, through hamster liver (Lushbaugh et al. 1978, Mattern et al. 1982). Unfortunately, the restoration of pathogenicity through *in vivo* passages has never been observed for previously cultured *N. pemaquidensis* (see

Kent et al. 1988, Morrison et al. 2005). The maintenance and propagation of *Neoparamoba pemaquidensis in vivo* is currently the only source of virulent isolates.

2.2.1 Methodology

A model of experimental infection was established by cohabitation of naïve fish with AGD-infected Atlantic salmon (see Munday et al. 2001). The major limitation with the cohabitation approach was that there was little control over the severity of the experimental infections. Under such conditions, the nature of infectious agents could not be accurately identified and other pathogens may have contributed to the disease. Zilberg et al. (2001) established an experimental model allowing more controlled AGD challenge: the infectious material harvested from the gills contained at least 97 % *Neoparamoeba* sp. and the severity of the disease was positively correlated with the initial concentration of amoebae in the inocula. Despite these modifications, this model still did not exclude the possibility of other organisms contributing to the disease and does not provide control over the kinetics and severity of the disease. Morrison et al. (2004) improved the reproducibility of the infection by selecting trophozoites by adherence to the bottom of a flask, thus insuring the overall viability of inocula. The minimum infective dose in this model to induce AGD (10-50 cells/ L) in Atlantic salmon is very similar to that observed in field conditions (Douglas-Helders et al. 2003), which may be beneficial in the future screening of vaccines and chemotherapeutants. Our laboratory established reliably AGD in Atlantic salmon by using the method described by Zilberg et al. (2001); the severity of the experimental infection could not be controlled and could vary throughout the year for unknown reasons.

In invertebrates, paramoebiasis may be maintained either by cohabitation between infected and naive individuals or by serial passage by inoculation. In blue crabs, successful experimental transmission of paramoebiasis was performed by inoculation of freshly sampled infected hemolymph into naïve crabs obtained from a paramoebiasis-free area (Johnson 1977). Disease was also successfully reproduced in clinically healthy lobsters that were held for one week with moribund lobsters from LIS that were naturally infected with *N. pemaquidensis* (Mullen et al. 2004). In green sea urchins, experimental transmission of the amoebae was performed by injection (Jones & Scheibling 1985) or by exposure to water containing diseased individuals (Scheibling & Stephenson 1984, Jones & Scheibling 1985). However, methods to maintain the parasite *in vivo* for long periods, to standardize the level of infection and to harvest the parasites have not been established.

2.2.2 Limitations of *in vivo* Maintenance

The use of live animals, e.g. Atlantic salmon and rainbow trout, as a culture system for virulent *N. pemaquidensis* is labor-intensive, time consuming and expensive. Before utilizing any experimental animals, an Animal Care Protocol form must be prepared and submitted to the Animal Care Committee justifying the scientific interest of the project, use, and welfare of experimental fish (CCAC 1993, 2005). This process has to be renewed annually. After the project is approved, the live system can be set-up and the animals ordered. The equipment used for holding aquatic animals (tanks, biofilters, mechanical filters, pumps, sterilisation equipment, pipes and monitoring instruments) is often complex and requires qualified personnel for set up and

maintenance. Experimental animals are generally obtained from a commercial source. The identification of each individual with a PIT (Passive Integrated Transponder) tag increases the initial price of the animals while permitting individual animal monitoring. Finally, the artificial sea water, which ensures greater environmental control, and the feed are expensive.

After the equipment is set up, the water is added and requires several weeks to stabilize. Saltwater tends to corrode the metallic parts of the equipment and its evaporation strongly affects the salinity level. Generally, a few fish are introduced and their survival is monitored to ensure that the system is ready to receive a larger group of animals. Aquatic animals are sensitive to water quality parameters and the latter must be continuously monitored. Very often, an alarm system is used to monitor system performance. Finally, when the population of fish is acclimated, the parasites can be introduced (the transmission of *N. pemaquidensis* is described in section 2.3.2). However, even with the optimal environmental parameters, the success of pathogen transmission can take a considerable period. After an infection is established, diseased fish must be regularly replaced to ensure continuous maintenance of the infection.

Another limitation of the *in vivo* culture system is the isolation of amoebae from the gills, which is time consuming (see section 2.1). Its success may be minimal, depending on the intensity of the infection, the bacterial contamination, and the putative overwhelming presence of other gill epibionts (organisms that live attached to another organism but without benefit or detriment to the host). Wang & Ahearn (2004) demonstrated that at densities of bacteria to *Acanthamoeba castellanii* of 100:1 or greater, the growth and survival of amoebae were inhibited. Since *N. pemaquidensis* and

associated bacterial fauna vary throughout the year with respect to morphological and biochemical features, it is impossible to obtain consistent pure gill derived isolates (Zilberg et al. 2001).

2.3 Culture of *Neoparamoeba pemaquidensis* on Tissue Culture Cells

Tissue or cell culture refers to techniques used to maintain living cells in an artificial medium and sterile environment, such as a Petri dish, flask or culture plate. The cells may be grown in media which closely approximate the *in vivo* environment. These techniques provide a simplified system in a controlled environment and provide a means of meeting commitments to the three R's (reduce, replace and refine) of animal welfare.

The use of cell lines has been highly successful in the culture and study of amphizoic amoebae. Some species hardly grow in conventional culture conditions: *Balamuthia mandrillaris* feed on tissue culture cells (monkey kidney and rat glioma cells) but do not feed upon bacteria (Schuster & Visvesvara 2004). *In vitro* cellular models of infection have also proven most successful in the collection of amoeba-derived cytolytic products. Some have been biochemically identified and the mechanisms of cytopathogenicity have been subsequently elucidated *in vivo* (Shin et al. 2000, Taylor et al. 1995). Cell culture systems have been used successfully to discriminate among pathogenic and non-pathogenic amoeba species or isolates (De Jonckheere 1980).

2.3.1 Methodology of Cell Culture System

Despite several descriptions of the cell-pathogen interactions in AGD (Roubal et al. 1989), most studies have been performed using histological sections, thus providing only structural information (Adams & Nowak 2003, Adams & Nowak 2004, Adams et al. 2004). Recently, a co-culture system of gill-isolated *N. pemaquidensis* grown on an Atlantic salmon gill cell line (RGE-2) was established to better understand this host-parasite relationship (Butler & Nowak 2004). A cytopathic effect (CPE) was observed: amoebae completely destroyed the monolayer within a few days. In addition, amoebae incubated with the RGE-2 cells exhibited a significantly higher growth rate over time than without cells. This study defined conditions (780 mOsm/ kg) suitable to maintain RGE-2 (remain viable for 7 days) and rapidly grow *N. pemaquidensis*. However, the initial inoculum of gill-isolated amoebae was not pure (> 98 % of *N. pemaquidensis*). The nature and possible effect of the remaining 2 % amoebae was unknown. In addition, the gill cell line, RGE-2, is not commercially available.

Our laboratory currently uses the method described by Lee et al. (2006), who pursued the co-culture approach of Butler & Nowak (2004) and tested different fish cell lines for the growth-supporting potential of cultured *N. pemaquidensis* (ATCC 50172). Amoeba growth was only observed on an established trout-derived cell line RTgill-W1 (Bols et al. 1994) in medium above 700 mOsm/ kg, which confirmed the importance of the osmolality level in the attachment of the parasites, as observed by Butler & Nowak (2004). Our laboratory tried the co-culture method in a medium at 640 mOsm/ kg: no amoeba growth was observed, confirming Lee et al.'s (2006) observations.

The ATCC 50172 isolate, which was originally obtained from AGD-infected coho salmon (Kent et al. 1988), has been maintained on artificial media for more than 18 years and is considered no longer to be pathogenic. Surprisingly, Lee et al. (2006) found that this isolate displayed CPE towards RTgill-W1 cells within 24 h and destroyed the feeder monolayer by 96 h (Figure 2.1).

Amoeba yield was as high as 5 x 10^5 amoebae/ mL following an initial inoculum of 10^4 amoebae/ mL. This is similar to the yield of *Balamuthia mandrillaris* grown on tissue culture in axenic conditions, i.e. 10^5 amoebae/ mL within 25-50 h (Schuster & Visvesvara 1996). By comparison, Martin (1985) reported a yield of 10^4 *Neoparamoeba pemaquidensis* (ATCC 30735) per millilitre after 18 days on artificial media.

Unlike Butler & Nowak (2004), Lee et al. (2006) established a model readily reproducible by using commercially available *N. pemaquidensis* (ATCC 50172) and the trout-derived cell line, RTgill-W1 (Bols et al. 1994). The RTgill-W1 cells are maintained in 75 cm^2 polystyrene tissue culture flasks containing Leibovitz medium (L-15) supplemented with 10 % foetal bovine serum (FBS). The cultures grow at room temperature (~ 20 °C) in gas exchange with air (Bols et al. 1994). Successful growth relies upon the brand of the FBS and culture flasks used. The RTgill-W1 must be adapted to higher osmolality levels in order to support the growth of *N. pemaquidensis*. After confluent monolayers are rinsed from L-15 medium (330 mOsm/ kg), they are kept in a mixture of L-15 20 % FBS and artificial sea water.

Over the course of 2-3 days, the saltwater is gradually increased in L-15 until making up 75 % of the total volume of the medium (750 mOsm/ kg).

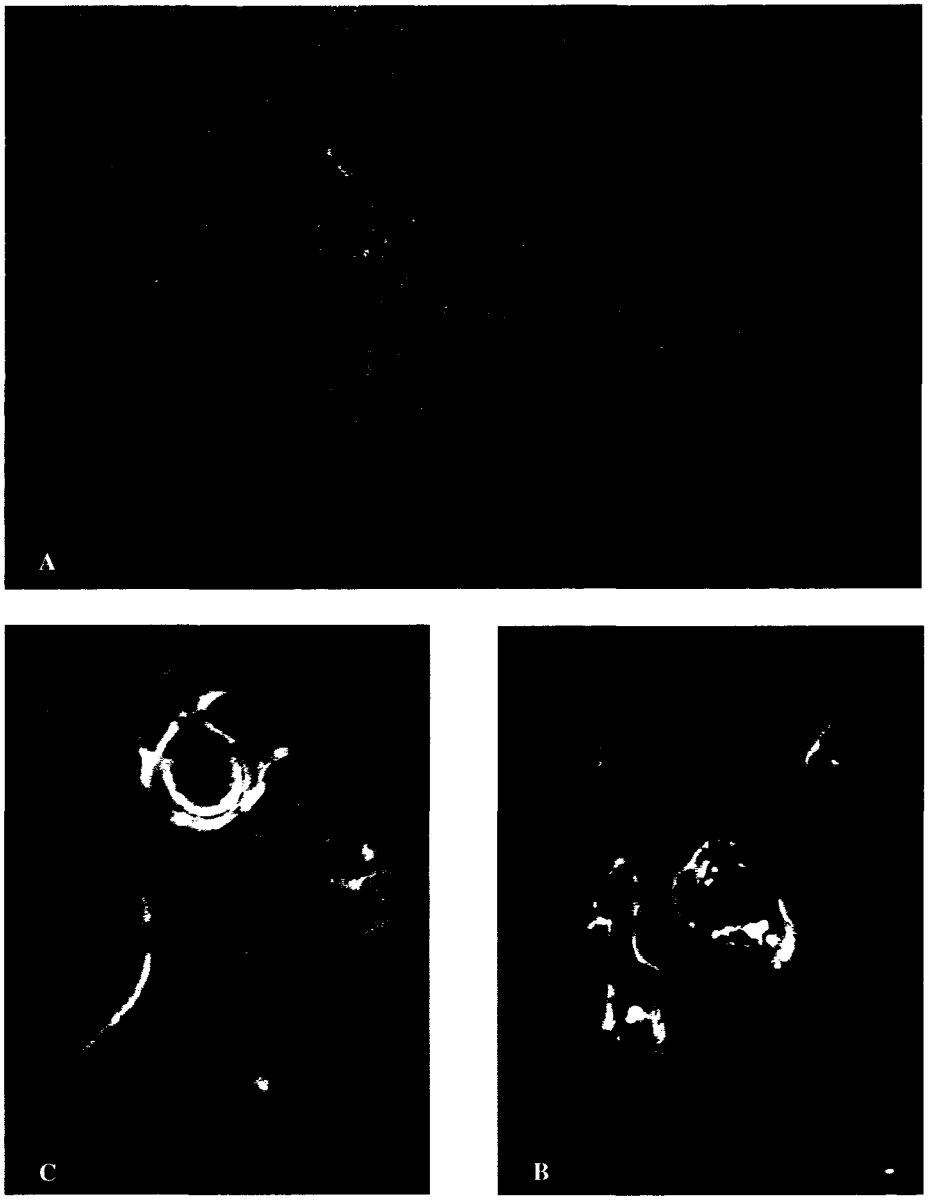

Figure 2.1. Cytopathic effects of *Neoparamoeba pemaquidensis* ATCC 50172 on RTgill-W1 monolayer at different magnifications (phase contrast microscopy). (**A**) RTgill-W1 monolayer (red frame) is progressively destroyed by amoebae (blue frame); lytic areas (orange frame) after 48 h (X 100); (**A**) is from Lee et al. 2006 and used with permission.(**B**). Attachment of amoebae (arrows) on RTgill-W1 cells (bar = 30 μm). (**C**) Amoeba consuming a RTgill-W1 cell (arrow) (bar =5 μm). N: nucleus; P: parasome

An inoculum of 10^4 amoeba/ mL is then added to the cell feeder layer. The co-culture is incubated at room temperature (~ 20 °C) for 4 days and monitored daily by phase contrast microscopy. Amoebae harvested after destruction of the monolayer exhibit more morphological similarities (increased size and more intracellular inclusions) with gill-isolated amoebae than with amoebae cultured on artificial media. ATCC 50172 harvested from RT-gill-W1 cells are larger than amoebae cultured on agar plates (Figure 2.2). Our observations are in agreement with the results of Lee et al. (2006), who found that amoebae grown in cell culture system were > 75 μm in diameter whereas amoebae from artificial medium were < 40 μm.

Lee et al. (2006) observed that amoebae harvested from RTgill-W1 cells had a slow division rate compared to their counterparts from artificial media. We subsequently tried to assess the influence of the type of medium and the origin (agar medium versus co-culture with RTgill-W1 cells) of the *N. pemaquidensis* ATCC 50172 on its division rate. *Neoparamoeba pemaquidensis* cultured on 994 agar medium were harvested after 4-7 days. *N. pemaquidensis* in co-culture with RTgill-W1 cells were harvested after 96 h (methods described above). Amoebae were then transferred to 25 cm^2 flasks containing various sea water-based media at ~ 20° C (75 % sea water/ 25 % Leibovitz medium, liquid ATCC 994 medium, and sea water). There were four flasks replicates per type of medium. Amoebae count was performed in all flasks every 24 hours during 7 days using propidium iodide and flow cytometry (see Chapter IV, section 4.2.3.2 and Appendix 2 for method). The results are presented Figure 2.3.

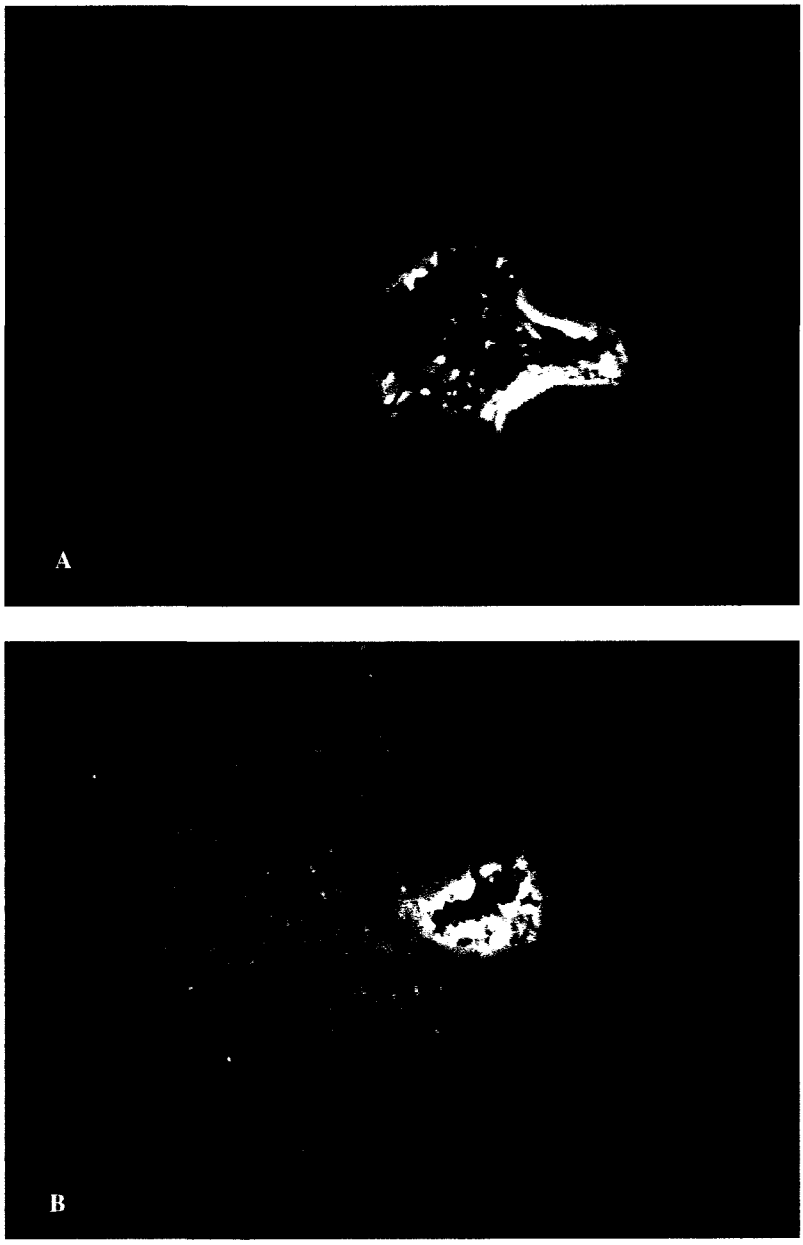

Figure 2.2. Size of cultured *Neoparamoeba pemaquidensis* under different culture conditions. (**A**) on RTgill-W1 cells and (**B**) in 75 % sea water/ 25 % L-15 (20% FBS) at 72 h. Bars = 10 μm; phase contrast microscopy.

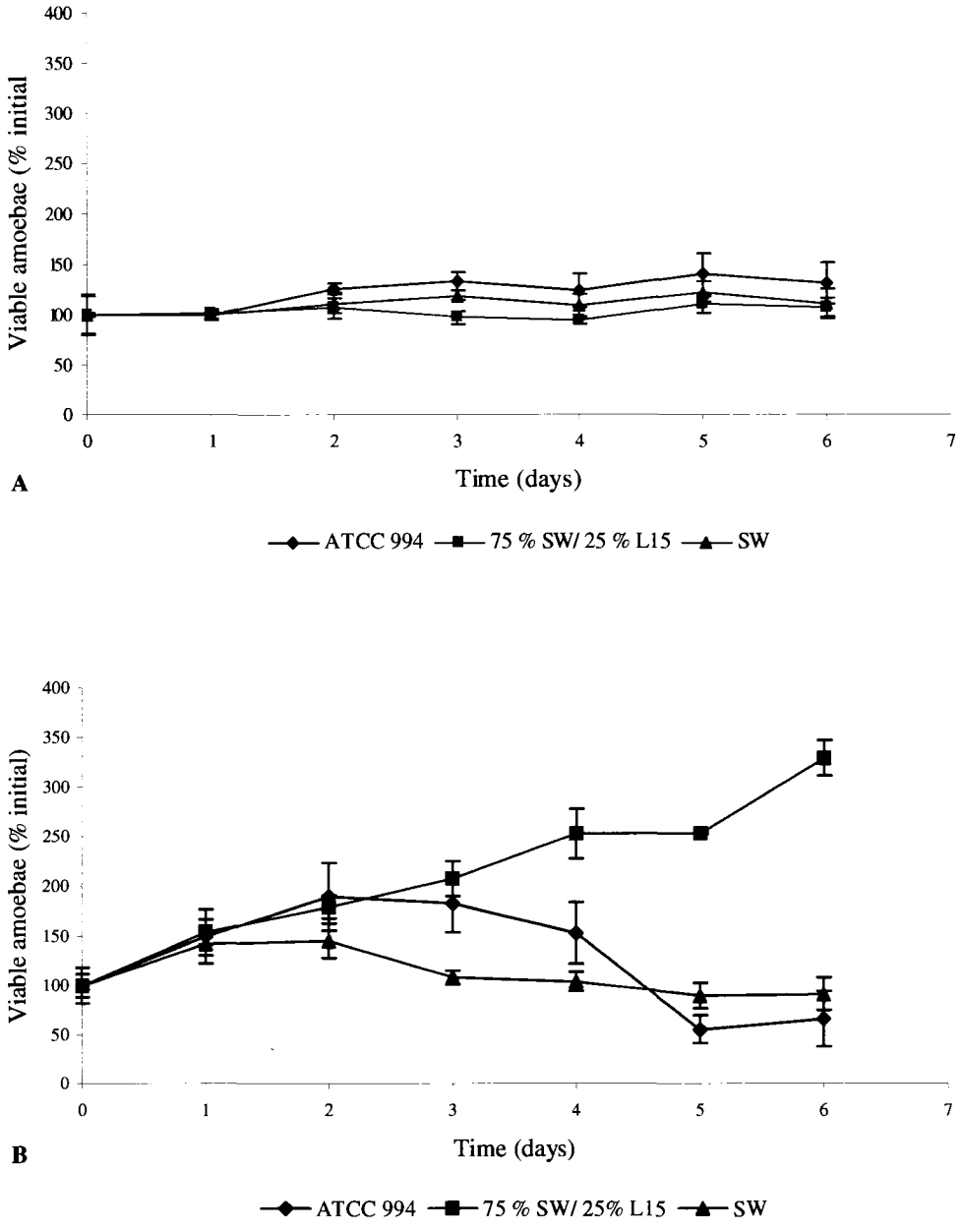

Figure 2.3 Growth of *Neoparamoeba pemaquidensis* ATCC 50172 in various sea water-based media. (**A**) amoebae were harvested from RTgill-W1 cells at 96 h;
(**B**) amoebae were harvested from 994 ATCC agar medium at 4-7 d.
Harvested amoebae were added to 25 cm^2 culture flasks, each containing 5 mL of various sea water-based media (ATCC 994, 75 % SW/ 25 % L15, and sea water). Counts of viable *N. pemaquidensis* (mean ± SD ; n = 4) were taken at 24 h intervals for 7 days using a flow cytometer (see Chapter IV, section 4.2.3.2 for method).
SW: sea water; L15: Leibovitz medium.

We observed that the division rate of *N. pemaquidensis* harvested from RTgill-W1 cells after ~ 96 h and placed in various sea water-based media was poor (Figure 2.3 A). In contrast, growth of *N. pemaquidensis* harvested from ATCC 994 agar medium after 4-7 days was stimulated after transfer to liquid media, especially in 75 % seawater/ 25 % L-15 20 % FBS (Figure 2.3B).

2.3.2 Limitations of Tissue Culture Methodology

Although the co-culture system of *N. pemaquidensis* and epithelial trout gill cells has recently been established (Butler & Nowak 2004, Lee et al. 2006), it requires further investigation. This technique has the potential to produce pure cultures of *Neoparamoeba pemaquidensis* in a short, predictable time period. It is presently difficult to precisely define limitations of this *in vitro* model; however some issues are predictable. First, fish cell lines, as with other cell lines, present several disadvantages (Freshney 1983). Maintenance of tissue cultures requires skill and expertise to diagnose problems with the culture as soon as they arise. Therefore, they must be envisioned as a continuous mode of culture and not be considered casually to run sporadic experiments. Cell lines require strict aseptic conditions and conscientious care. They also display marked preferences for certain additives and containers. For instance, our laboratory encountered major difficulties by trying new additives (serum) and flasks. Most cell lines, cell culture media and additives (i.e. serum) are expensive. Fortunately, cell line cryopreservation limits the number of routine cultures required for ongoing maintenance and provides a good back up in the event of contamination. Cryopreserved cell lines also

provide a source of cultures to return to periodically to minimize the potential for genetic drift in the routinely passaged cultures.

Secondly, cultures of *N. pemaquidensis* on RTgill-W1 cell line are not axenic: amoebae harvested from agar medium are associated with bacteria and therefore, contaminate the cell culture system when inoculated. The use of antibiotics may lower the bacterial load in the medium but is inefficient against endocytic bacteria, which subsequently spread in the cell culture system. The bacterial load associated with *N. pemaquidensis* harvested from agar medium is heavier than what may be observed in the co-culture system. The possible side effects of the bacteria on the co-culture system cannot be easily assessed although they should not be overlooked.

Finally, we observed that the level of cytopathic activity of ATCC 50172 varies for unexplained reasons. This has already been shown for other amoebae. De Jonckheere (1980) reported that the cytopathogenic activity of several strains of *Acanthamoeba* spp. was strongly influenced by temperature. Also, it may greatly depend on the target cell line. *Naegleria fowleri* shows variable degrees of cytopathogenicity for several mammalian cell lines but exhibits the strongest CPE toward the African green-monkey cell line (John & John 1989). Lee et al. (2006) showed that *N. pemaquidensis* ATCC 50172 grew preferentially on RTgill-W1 compared with the nine other fish cell lines tested. Additionally, the displayed cytopathogenicity toward gill cells does not prove that the *N. pemaquidensis* is pathogenic *in vivo*. Current knowledge on other amphizoic amoebae such as *Acanthamoeba* spp. and *Naegleria* spp. growing on feeder monolayers will be helpful in the understanding of this newly established cell culture system.

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III. INDUCTION OF AMOEBIC GILL DISEASE IN TWO SALMONIDS BY EXPOSURE TO *NEOPARAMOEBA PEMAQUIDENSIS* HARVESTED FROM RAINBOW TROUT GILL CELL CULTURES

3.1 Introduction

Neoparamoeba pemaquidensis Page, 1987 is an ubiquitous marine amoeba that can be isolated from the environment (Page 1970, as *Paramoeba pemaquidensis*). Disease outbreaks in marine invertebrates have been attributed to *N. pemaquidensis* for the American lobster, *Homarus americanus* (see Mullen et al. 2004, Mullen et al. 2005) and green sea urchins, *Strongylocentrotus droebachiensis* (see Jones 1985, as *Paramoeba invadens*). Also, this amoeba is considered to be the primary cause of amoebic gill disease (AGD) in sea-farmed salmonids (Kent et al. 1988, Roubal et al. 1989, Munday et al. 1990, Zilberg & Munday 2000, Adams & Nowak 2003, Adams & Nowak 2004), although Koch's postulates have not yet been fulfilled.

Koch's postulates were described in 1890 by the German physician and bacteriologist Robert Koch as criteria to be met to establish whether a given pathogen is the cause of a given disease. Koch's postulates are as follows: 1) the specific microorganism should be present in all cases of animals suffering from a specific disease; 2) the specific microorganism should be isolated from the diseased animal and grown in pure culture on artificial laboratory media; 3) this freshly isolated microorganism, when inoculated into a healthy animal, should cause the same disease seen in the original animal; 4) and the microorganism should be reisolated in pure culture from the experimental infection (Koch 1891).

Neoparamoeba pemaquidensis was consistently isolated from sick fish (Howard & Carson, 1993, Dyková et al. 2000), although several different organisms have been

isolated from gills of fish with AGD either with or without *N. pemaquidensis* (Bermingham & Mulcahy 2004, Bermingham & Mulcahy 2006). Additionally, Jones (1985) and Jones & Scheibling (1985) demonstrated that *N. pemaquidensis* (formerly *Paramoeba invadens*) was consistently isolated from moribund green sea urchins.

Pure cultures of *N. pemaquidensis* can be successfully grown on sea water-based artificial media (Table I, Chapter II). However, attempts to experimentally elicit AGD in Atlantic salmon using *Neoparamoeba* cultured on artificial media have never been successful (Kent et al. 1988, Howard & Carson 1993, Morrison et al. 2005). Transmission of AGD has been achieved through co-habitation of AGD infected salmon with naive salmon (Akhlagi et al. 1996, Zilberg et al. 2001). Infection may also be induced by exposure of fish to crude gill preparation or *Neoparamoeba* freshly harvested from infected fish (Zilberg et al. 2001).

Several pathogens do not integrate well with the paradigm stipulated by Koch's postulates. Many obligate parasites such as viruses, which multiply by usurping cellular metabolic pathways, cannot be grown in cell-free-media as required in the second postulate. In the case of *N. pemaquidensis*, it is not yet possible to fulfill the third postulate as cultured isolates rapidly lose their infectivity (defined as a measure of the ability of a disease agent to establish itself in a host; virulence is defined as the ability of any infectious agent to produce disease; this term is synonymous with pathogenicity) on artificial media for unknown reasons. Douglas-Helders et al. (2003) demonstrated that *N. pemaquidensis* isolated from gills were still infective after 14 days on artificial medium, while Morrison et al. (2005) showed that amoebae harvested from gills could not induce AGD after 34 days in culture. However, Jellett & Scheibling (1988)

maintained the pathogenicity of *N. pemaquidensis* to green sea urchins from 15 to 58 weeks on artificial media, depending on the bacterial food source.

The strict application of the original Koch's postulates can lead to failure to consider the specific requirements of a given pathogen, thus making proof of disease causation extremely challenging. Fredericks & Relman (1996) extensively debated the necessity to revise and adapt them, and use technological advances such as genotype-based microbial identification to prove the involvement of an agent in the disease. New approaches to *in vitro* culture of *Neoparamoeba pemaquidensis* have recently been undertaken. Butler & Nowak (2004) grew freshly gill-isolated amoebae from Atlantic salmon on a gill-derived Atlantic salmon cell line, and Lee et al. (2006) grew long-term cultured avirulent amoebae (ATCC 50172) on a rainbow trout cell line (RTgill-W1). In both studies, the amoebae were cytopathogenic and rapidly destroyed the cell monolayers

The correlation of cytopathogenicity *in vitro* and virulence *in vivo* has been observed many times in amoebic diseases (Mattern & Keister 1977, Shin et al. 2000, Schuster 2002). Therefore, the objective of this study is to explore the possibility that avirulent *Neoparamoeba pemaquidensis* ATCC 50172 grown on a gill-derived feeder layer may have recovered their initial pathogenicity towards susceptible hosts, i.e. Atlantic salmon and rainbow trout.

3.2 Materials and Methods

3.2.1 Cell Cultures

3.2.1.1 Cultures of *Neoparamoeba pemaquidensis*

Neoparamoeba pemaquidensis 50172 was obtained from the American Type Culture Collection. *Neoparamoeba pemaquidensis* was cultured on sea water-based agar medium ATCC 994. This latter was prepared according to the formula described in Appendix 1 and steam autoclaved at 121 °C for 20 min. When cooled to 40 °C, the agar was poured into Petri dishes (Ultident). Plates were left overnight at room temperature (~20 °C) to solidify. They were then covered with a thin lawn of *Klebsiella pneumoniae* and incubated at 37 °C for 24 h. After cooling, 500 µL of amoeba suspension, harvested by gentle and repeated flushing from 7-10 d old established cultures, were poured and spread onto the plates. Finally, amoeba culture plates were sealed with parafilm to prevent evaporation and kept in a dark cupboard at room temperature (~20 °C). Subculturing was performed every 10 d.

3.2.1.2 Cultures of Rainbow Trout Gill Cells

The rainbow trout gill cell line RTgill-W1 was continuously cultured in 75 cm^2 polystyrene cell culture flasks (Sarstedt) as described by Bols et al. (1994). Cells at passages 70-75 were used in this study. Cells were maintained at room temperature (~ 20 °C) in 10 mL Leibovitz-15 medium (Sigma) supplemented with 10 % foetal bovine serum from Gibco (L-15 10 % FBS) and a solution of L-glutamine (10 mM), penicillin (5000 IU/ mL), and streptomycin (5000 µg/ L) (Gibco). The RTgill-W1 cells were subcultured by removing the medium, rinsing the monolayer with

ethylenediaminetetraacetic acid (EDTA, Versene) and detaching the monolayer by incubation with trypsin (Sigma). Cells were resuspended in 20 mL L-15 with 10 % FBS and dispensed into two new 75 cm^2 cell culture flasks (10 mL/ flask).

3.2.2 Fish Source and Husbandry

Sea water acclimated Atlantic salmon, *Salmo salar*, (~ 470 g ± 100 g; 39 cm ± 2.8 cm total length) were obtained from the Centre for Aquatic Health Sciences (University of PEI, Canada). Rainbow trout, *Oncorhynchus mykiss*, (100-300 g and 20-30 cm total length) were obtained from Cardigan Fish Hatchery (PEI, Canada).

The fish housing system comprised three 0.75 m diameter x 0.4 m deep circular, dark green, fibreglass reinforced plastic tanks, each containing approximately 80 L of sea water, capable of holding approximatively 2.5-3 kg of Atlantic salmon, and a 200 L pumping reservoir. Mechanical filtration was carried out by a two stage filtration system. The primary system took place within the tank via a patented double drain system. Sea water was supplied by a recirculating water system consisting of a 300 L capacity plastic bead biofilter with a total system of 750 L. The filter is operated as a side loop from the main pump. Outflow from this filter returned directly to the reservoir. The water temperature was controlled by a water cooled titanium chiller. Flow in the tanks was maintained by a single 0.5 hp Starite centrifugal pump. Approximately 6 L/ min was continuously delivered to each tank. No UV sterilizer or activated carbon were employed in the process. The air supply was from a 0.25 hp Sweetwater regenerative blower. Tanks, reservoir and biological filter were supplied by Waterline Ltd of Winsloe, PEI, Canada.

Fish were introduced and acclimated to the recirculated artificial seawater (Instant Ocean®) over 2-3 months before challenge. All tanks were held at water temperature of 16 °C and salinity of 32.7 ‰. The circadian cycle was preset on 12 h light and 12 h dark. Fish were fed commercial dry feed (Corey Feed Mills Ltd, NB, Canada) and monitored once daily. Prior to exposing the fish to the *Neoparamoeba*, a subsample of three fish was euthanized and a necropsy was performed to document the health status of the fish and, in particular, to establish the absence of any amoeba infection of the gills.

For this experiment, 1 tank contained 4 long-term system-acclimated Atlantic salmon and a second tank contained 3 long-term system-acclimated Atlantic salmon and 1 rainbow trout. The third tank contained 2 long-term system-acclimated rainbow trout and 3 Atlantic salmon, which were added the day of the challenge.

3.2.3 Experimental Transmission

3.2.3.1 Incubation of *Neoparamoeba pemaquidensis* with Epithelial Gill Cells

Confluent rainbow trout gill-derived cell monolayers (95 %) were rinsed with L-15 10 % FBS medium (osmolality 330 mOsm/ kg) and were adapted to higher osmolality by addition of a mixture of L-15 20 % FBS and artificial seawater. The artificial sea water was made from artificial sea salts (Instant Ocean®) and sterile water, and was sterilized by filtration through a 0.2 µm membrane filter. The proportion of artificial sea water started at 25 % (440 mOsm/ kg) on Day 1 and increased to 50 % (560 mOsm/ kg) on day 2. On Day 3, it reached 75 % (780 mOsm/ kg). Approximately 10^4

amoebae harvested from agar plates and suspended in artificial sea water were then added to each cell culture flask. A control flask containing only gill cells was also prepared to monitor the adaptation of gill cells to high osmolality. The co-culture flasks were inspected daily by phase contrast illumination on a Nikon Diaphot-TMD inverted microscope.

3.2.3.2 Preparation of the Amoeba Inoculum

When the RTgill-W1 monolayer was nearly totally destroyed (~ 96 h), 20 mL artificial sea water (30-32 ‰ salinity) was added for 2-3 h to kill surviving RTgill-W1 cells and remove gill cell debris. All the culture flasks were emptied and the adherent amoeba cultures were rinsed of gill cell debris. A small volume of artificial sea water (10 mL) was then added to a culture flask. This latter was tapped vigorously to release and suspend amoebae. This suspension was added to a second culture flask and the process was repeated. The transfer from flask to flask allowed for the gradual increase in the concentration of amoebae without the need for centrifugation.

The concentration of amoebae in the final 10 mL suspension was determined using a hemacytometer (Neubauer) and viability was immediately assessed using cell absorption of 0.5 % trypan blue (Sigma) in artificial seawater (~ 20°C). To perform the cell count and viability determination, a 20 µL aliquot of amoeba suspension was mixed with an equal volume of trypan blue solution in artificial seawater. A 10 µL volume was transferred to each chamber of the hemacytometer and the amoebae counted at X 250 (light microscopy). The count was repeated once.

3.2.3.3 Immersion Exposure to *Neoparamoeba pemaquidensis*

First, the Atlantic salmon from each tank (3 at a time) were successively transferred into a tote containing tricaine methanesulfonate (150 mg/ L). After fish were anaesthetized, they were rapidly rinsed in a tote filled with clean artificial sea water to remove any tricaine methanesulfonate. Finally, each group of fish was allowed to recover (~10 min) in a static tote inoculated with various concentrations of *N. pemaquidensis* harvested from RTgill-W1 cell cultures before returning to their tank. The group consisting of 3 long-term system-acclimated Atlantic salmon and the 3 newly introduced Atlantic salmon were exposed to 1,000 amoebae/ L. The group of 4 long-term system-acclimated salmon was exposed to 10,000 amoebae/ L. The rainbow trout were not transferred from the tank to the totes. After the Atlantic salmon were returned to their tank, the tote (12 L of sea water) containing the suspension of amoebae (2×10^6 *N. pemaquidensis*) was added to the 800 L capacity tank system, resulting in a final concentration of 2,500 amoebae/ L.

3.2.4 Fish and Water Sampling Procedures

3.2.4.1 Fish Sampling

Fish were sampled at 34, 48 and 98 d post-exposure. At each interval, a subsample of 3 fish was caught by crowding and dip-netting. Following capture, fish were killed by overdosing with tricaine methanesulfonate (100 mg/ L) or by manual stunning. A complete necropsy was performed, with special attention to the gills. Fish gills were examined and lesions rated using the Tassal Pty Ltd (Australia) gross gill scoring method for amoebic gill disease detection (Powell et al. 2001) (Table II).

Table II. Tassal* P/L gross AGD lesion scoring scheme

Infection level	Score	Gross signs
Clear	0	Gills appear clean, healthy red colour
Very light	1	1 mucoid patch, light mucus accumulations
Light	2	2-3 mucoid patches, some paling colour
Medium	3	Established thickened mucoid patches and mucus
Heavy	4	> 3 mucoid patches or a single large patch resulting from patch fusion

* Tassal Group Ltd is a salmon farming company in Tasmania, Australia.

The second and third gill arches were collected and immediately fixed in 10 % seawater formalin for subsequent processing for histology. After processing and staining with haematoxylin and eosin (Adams et al. 2004) and Giemsa (currently used in our laboratory), the 5 μm section slides were examined using a Zeiss Axioplan 2 microscope (X 400 and 1,000 magnifications). The remaining gill arches were excised, cut in pieces and placed in a plastic tube (Falcon) containing 15 mL of artificial sea water (32 ‰ salinity) supplemented with antibiotics [5 % v/v 5000 UI/ mL penicillin and 5 mg/ mL streptomycin (Gibco), 1 % v/v 10 mg/ mL gentamycin (Sigma)] at 20 °C (Butler & Nowak 2004). The gills were kept for about 30 min in sea water (~ 20 °C) until processing. These preparations were used to isolate and culture amoebae. Fish that died during the trial were removed and examined as described above.

3.2.4.2 Water Sampling

Fish housing system water was sampled before and after the addition of *N. pemaquidensis* into the tanks to check for the presence and viability of the parasites. Water from the 3 fish housing tanks, the biofilter and the reservoir was sampled and cultured in 25 cm^2 sterile cell culture flasks (Falcon) at room temperature (~ 20 °C) to attempt isolation of *N. pemaquidensis*. Also, the initial exposure inoculum was sampled to assess the viability of the amoebae used for exposure. In total, 20 flasks (2 flasks/ sampling location), each containing 5 mL sampled water only, were monitored daily.

3.2.4.3 Isolation of *Neoparamoeba pemaquidensis* from Gills

The artificial seawater-antibiotic solution containing gill arch pieces was vigorously agitated manually for 5 min and the supernatant transferred to 25 cm^2 culture flasks (Falcon). Amoebae were allowed to settle for 2 h. Adherent amoebae were gently rinsed with artificial seawater and 2 mL seawater was added to the flask. The presence of parasome-containing amoebae was assessed using a phase contrast Nikon Diaphot-TMD inverted microscope.

Detachment of the amoebae was performed by tapping the flask against a rubber pad. The suspension was harvested and 20 µL aliquots were poured onto sea water-based agar medium. Cultures were kept at room temperature (~ 20 °C) and monitored daily using phase contrast microscopy.

Repeated selection of amoeba populations led to more homogeneous cultures. Individual cells were then cut out from the agar and transferred face down on fresh agar plates. The presence of parasomes was verified at each purification step. When parasome-containing amoebae were isolated, PCR was used for identification as described by Caraguel (2006).

3.3 Results

3.3.1 *Neoparamoeba pemaquidensis* Recovery from Fish and Water Samples

3.3.1.1 Fish Samples

The fish used in this experiment were naive to AGD. Full necropsies performed on three long-term, housing system acclimated, Atlantic salmon revealed healthy gills. Neither hyperplasia and excessive mucus nor parasites were observed during both gross and microscopic examinations.

Results of the transmission trial are presented in Table III. Full necropsies were performed on every fish. One salmon (Fish n°1) died 3 d after the start of the trial but this individual was injured during exposure to the amoeba and its ventral skin exhibited serious haemorrhages. One trout and one salmon died respectively at 11 and 82 d post-exposure of undetermined causes.

Neither gross or microscopic signs of AGD (all AGD scores = 0) were detected at 34, 48 and 98 d post-exposure in fish exposed to the amoebae. Gills were healthy and no bacterial proliferation was observed.

3.3.1.2 Water Samples

No *Neoparamoeba pemaquidensis* was recovered from the fish housing system water before exposure of the salmon to *Neoparamoeba*. In contrast, *N. pemaquidensis* were recovered from the fish housing system water after the exposure challenge. Amoebae from the immersion exposure bath and from the water of the inoculated fish housing system survived in cell culture flasks for a minimum of 10 d.

Table III. AGD lesion score, culture, and histology results of the transmission study

Fish identification number	Species S: Salmon T:Trout	Gender M:Male F: Female	Weight (g)	Length (cm)	Tank number	Euthanasia Y:Yes N: No	Time to death (days)	AGD lesion score	Culture & histology results N:Negative P:Positive
1	S	M	493.2	40.0	6	N	3	0	N
2	T	F	111.6	21.0	4	N	11	0	N
3	S	F	352.8	34.5	6	Y	34	0	N
4	S	M	335.4	33.5	5	Y	34	0	N
5	S	F	384.8	39.7	4	Y	34	0	N
6	S	M	645.5	40.6	4	Y	48	0	N
7	S	F	638.5	44.5	5	Y	48	0	N
8	S	M	568.7	39.7	6	Y	48	0	N
9	T	NR	NR	NR	6	N	82	0	N
10	S	M	472.1	39.7	5	Y	98	0	N
11	S	F	490.7	39.4	5	Y	98	0	N
12	S	M	362.7	36.5	4	Y	98	0	N
13	T	M	335.1	31.0	4	Y	98	0	N

NR: Not recorded

3.3.2 Isolation of *Neoparamoeba pemaquidensis* from Gills

None of the gill tissues cultured on artificial sea water liquid medium yielded *N. pemaquidensis*. A few *Vannella*-like amoebae were observed.

3.4 Discussion

The establishment and development of AGD results from an interplay among environmental conditions, fish immune response and gill health, and density and infectivity of *Neoparamoeba pemaquidensis*. In this section, possible reasons explaining the non-transmission of *N. pemaquidensis* harvested from RTgill-W1 cells to naive Atlantic salmon and rainbow trout are systematically reviewed and discussed.

The environmental parameters used in this study are appropriate as they have proven successful in the induction and transmission of *N. pemaquidensis* to Atlantic salmon and rainbow trout, by cohabitation. Similar tank set-up, water quality and commercial sources of experimental fish are being used by our research team for the continuous *in vivo* culture of virulent *N. pemaquidensis* and are considered as a control system in this experiment.

Atlantic salmon is an ideal model in AGD research because it is the most susceptible species to *N. pemaquidensis* (Nowak 2000). Also, it seemed important to verify if the amoebae harvested from a rainbow trout gill-derived cell line had developed a marked preference toward rainbow trout over the Atlantic salmon. To determine whether stressed fish would be more susceptible to the infection, 3 non system acclimated Atlantic salmon were used for the amoeba challenge.

Generally, methods to harvest amoebae from gill tissues could cause cell damage and may kill the amoebae. Consequently, abrupt temperature variation, vortexing, centrifugation, and gill scraping were cautiously avoided during harvesting of amoebae. The use of freshwater to detach the amoebae from the gill by osmotic shock and use of EDTA/ trypsin solution to suspend adherent cells were also avoided. The harvesting method of cultured amoebae was restricted to vigorous flask tapping and gentle rinsing (Martin 1985). Only amoebae selected by plastic adherence (Morrison et al. 2004), were used to inoculate the fish-holding system. The inoculum and inoculated sea water were sampled and cultured to verify amoeba viability after the exposure challenge. Therefore, the amoebae used in this study were most likely vigorous.

Morrison et al. (2004) refined the AGD infection model by improving the purification of the gill-isolated inoculum and the minimum infective dose, i.e.10 amoebae/ L, and suggested that the severity of AGD depends on the concentration of amoebae. In this study, the purity of the *N. pemaquidensis* (ATCC 50172) inoculum was 100 %. Fish were anaesthetized to reduce the gill flow, thus facilitating the adhesion of amoebae on gills. During the recovery period, they were placed into water containing high numbers of parasites (1,000 and 10,000 amoebae/ L). Finally, amoebae were inoculated into fish holding tanks at concentrations largely in excess (2,500 amoebae/ L) of that routinely used to induce experimental AGD with amoebae freshly harvested from gills (Zilberg et al. 2001, Morrison et al. 2004). Additionally, the duration of the exposure period (> 30 days) largely exceeded the time usually required for development of clinical AGD in the control system (~10-20 days). Dose and exposure duration were higher and longer than what was described in previous studies (Zilberg et al. 2001,

Morrison et al. 2004), suggesting that our study design should have facilitated successful transmission of *N. pemaquidensis*.

The choice of the isolate, *Neoparamoeba pemaquidensis* ATCC 50172 followed from the study of Lee et al. (2006). The current collection of *Neoparamoeba* spp. is limited to a few species and does not include any pathogenic strain. This is in contrast with what is observed in the most studied genera of amoebae (i.e. *Acanthamoeba* and *Naegleria* spp.); they usually consist of both virulent and avirulent strains. For example, the genus *Acanthamoeba* has been classified into 13 different genotypes using rDNA sequences (Gast 2001). Sequence analysis revealed that 95 % of *Acanthamoeba* isolates producing keratitis belong to one genotype, suggesting that pathogenicity may be limited to certain genotypes. A recent assessment of *N. pemaquidensis* has been able to separate subspecies (Caraguel, 2006). However, before losing its virulence on artificial media, *Neoparamoeba pemaquidensis* ATCC 50172 was initially pathogenic as it was isolated from AGD affected coho salmon (Kent et al. 1988).

Hu et al. (1992) showed that induction of gene activity correlating with virulence occurred when *Naegleria fowleri* fed on tissue culture cells but not when they fed on bacteria or when they were grown in axenic culture. In this study, *N. pemaquidensis* underwent morphological changes and rapidly destroyed the feeder layer when placed into the rainbow trout gill cell-culture system, but they were non infective *in vivo*. The long-term culture on agar has probably contributed to down-regulation of certain factors implicated in *N. pemaquidensis* infectivity and AGD pathogenesis. This would be in agreement with Phillips (1973), who demonstrated that *Entamoeba histolytica* did lose

its infectivity after more than 2 years maintenance in axenic culture *in vitro*, and its virulence could not be restored after passage in hamsters.

Hu et al. (1991) observed that loss of virulence correlated with loss of pathogenic protein synthesis patterns in axenically grown amoebas. Amoeba adherence to host cells is a prerequisite for colonization. *Acanthamoeba* binding to epithelial corneal cells, which is mediated by lectin, is a mandatory step in producing keratitis and avoiding being washed out by tears (Garate et al. 2004). The observation that no *N. pemaquidensis* was found with histology or in culture may suggest that adhesion on gill tissues could not occur *in vivo*. However, *N. pemaquidensis* 50172 displays significant tropism toward rainbow trout gill cells *in vitro* (Lee et al. 2006). Anti-acanthamoebic IgA antibodies inhibit the binding of *Acanthamoeba* to host cells, block the secretion of cytotoxic substances, and protect against *Acanthamoeba*-mediated keratitis in an *in vivo* model (Ferrante 1991, Leher et al. 1999). Therefore, host factors may prevent adhesion of the *N. pemaquidensis* on the gills in this study.

The origin, variety, and abundance of the bacterial food source provided to cultured amoebae apparently are important in the pathogenesis of AGD. In this study, marine amphizoic amoebae were fed with bacteria (i.e. *Klebsiella pneumoniae* or *Enterobacter aerogenes*) that normally are not their usual food source in their original environment. Inadequate quality of food during *in vitro* culture has been associated with loss of virulence of the *N. pemaquidensis* isolate causing mortalities in green sea urchins (Jellett & Scheibling 1988). When cultured on a monoxenic lawn of *Pseudomonas nautica*, *N. pemaquidensis* lost its virulence after 15 weeks. When cultured on a polyxenic lawn of marine bacteria, loss of virulence occurred after 58 weeks (Jellett &

Scheibling 1988). Lee et al. (2006) observed that amoebae harvested from RTgill-W1 cell cultures were less cytopathogenic than those obtained from bacterized agar plates. This is in agreement with the observations of Bracha & Mirelman (1984), who demonstrated that preincubation of *Entamoeba histolytica* with increasing amounts of *Escherichia coli* gradually increased the rate of destruction of mammalian cell monolayers. Replacing the current bacterial food source (*Klebsiella pneumoniae* and *Enterobacter aerogenes*) of *N. pemaquidensis* with more appropriate sources (polyxenic and/or monoxenic marine lawns of bacteria), should be the next step in assessing the role of bacteria in amoeba pathogenicity.

The association of bacteria-amoeba has been observed in several paramoebiasis cases. Bowman & Nowak (2004) identified the marine bacterium *Psychroserpens* as a potential opportunistic pathogen associated with salmonid AGD. Noble et al. (1997) suggested that freshwater amoebic disease of rainbow trout gills was also associated with bacterial infection. However, the nature of this relationship remains obscure. Bacteria tend to proliferate around the net-pens as the water contains high levels of dissolved nutrients released by the fish and fish feed. Proliferating bacteria colonize gills, and their presence and/or the lesions they induce may attract and provide opportunity for pathogens such as *N. pemaquidensis* to multiply. Marciano-Cabral & Cline (1987) showed that *Naegleria fowleri* preferred bacteria over nerve cell extracts *in vitro*. Another hypothesis would be that *N. pemaquidensis* is non pathogenic, but harbour harmful bacteria and transmits them when amoebae adhere to host tissues. Subsequently, production of mucus concurrent to bacterial proliferation would allow a ready food source and cause the amoebae to bloom and physically damage the gills.

More likely, intra-protozoal growth of bacteria may optimize the amoeba potential for virulence. Fritsche et al. (1998) investigated the possible role of different bacterial endosymbionts in acanthamoebic pathogenesis and reported that all endosymbiont-infected amoebae produced statistically significant enhancement in cytopathogenicity in comparison to endosymbiont free amoebae. Embar-Gopinath et al. (2005) showed that the presence of a *Winogradskyella* sp., a gram negative bacterium phylogenetically close to *Psychroserpens* (Nedashkovskaya et al. 2005), enhanced the pathogenicity of *Neoparamoeba* sp. *in vivo*. In contrast, fish exposed to both *Neoparamoeba* sp. and *Staphylococcus* sp. did not develop exacerbated AGD infection (Embar-Gopinath et al. 2005).

The suggestion that *N. pemaquidensis* may need a specific bacterial "partner in crime" to elicit AGD has never been considered to elaborate optimal *in vitro* culture conditions. Studies on requirements for *Neoparamoeba* virulence stimulation by bacterial endosymbionts should focus on bacteria species, incubation time of amoebae with bacteria, bacteria growth phase, and impact of chemical or physical treatments undergone by bacterial populations (i.e. heat-inactivation, sonication). In contrast, Roubal & Lester (1989) observed that no large bacterial loads were present prior to the presence of *Neoparamoeba* sp., indicating that the amoebae may be primary pathogens as previously suggested by Kent et al. (1988). The question whether *N. pemaquidensis* is an opportunistic pathogen or the primary agent of paramoebiasis has been raised elsewhere, especially during the LIS die-off (Mullen et al 2004). Dyková et al. (1999) questioned the role of free-living amoebae other than *Neoparamoeba* sp. in the pathogenicity of AGD. Leiro et al. (1998) reported species belonging to the genera

Platyamoeba and *Vannella* from AGD-infected turbot. In Ireland, high numbers of histophagous scuticociliates, *Ichthyobodo*-like flagellates and various species of amoebae other than *Neoparamoeba* sp. were observed in close association with AGD gill pathology, suggesting that *Neoparamoeba* alone was not the primary agent of the disease (Bermingham and Mulcahy 2004, Bermingham and Mulcahy 2004).

This pilot study did not experimentally induce AGD in Atlantic salmon and rainbow trout by exposure to *N. pemaquidensis* 50172 recovered from RTgill-W1 cell cultures. The issues raised by these results deserve further detailed studies, and many questions need to be answered. One of the most important is to assess whether pathogenic gill-detached *N. pemaquidensis* may conserve their virulence when continuously cultured on a gill-derived cell line. Also, further research on culture conditions necessary to the maintenance and/or restoration of virulence should be undertaken. The understanding of the intriguing relationship between bacteria and *N. pemaquidensis* is probably the key to successful fulfillment of Koch's postulates for paramoebiasis.

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IV. *IN VITRO* EFFICACY OF AMOEBICIDAL COMPOUNDS AGAINST *NEOPARAMOEBA PEMAQUIDENSIS*

4.1 Introduction

Paramoebiasis caused by the amphizoic amoeba *Neoparamoeba pemaquidensis* is a significant disease of several commercially important aquatic animal species. Epidemics of paramoebiasis have sporadically occurred in green sea urchins (*Strongylocentrotus droebachiensis*) populations in Atlantic Canada (Jones 1985), blue crabs (*Callinectes sapidus*) in Virginia, USA (Sprague & Beckett 1968), and the disease is believed to have had an important contribution to the die-off of American lobsters (*Homarus americanus)* in Long Island Sound, USA in 1999 (Mullen et al. 2004). Amoebic gill disease, caused by *N. pemaquidensis* of farmed Atlantic salmon (*Salmo salar*) is a major health issue for farmed Atlantic salmon with up to 20 % losses annually attributable to this disease (Munday et al. 2001). Current treatment for the disease in marine fish farms consists of freshwater baths. There is a lack of effective alternative treatment options. With time, outbreaks tend to become more severe and have extended throughout the year (instead of the warm season). This has resulted from increased resistance of *N. pemaquidensis* to freshwater treatment.

In vitro assays are useful for screening chemotherapeutants and disinfectants before testing on live animals. In this context, a robust and standardized test for determination of *N. pemaquidensis* viability would be useful. Viability determination of *N. pemaquidensis* is normally undertaken by the trypan blue exclusion test (Powell & Clark 2003, Powell et al. 2003). This vital dye does not react with the cell unless the membrane is damaged. Therefore, all the cells which exclude the dye are viable (Freshney

1987). This method requires tedious microscopic observation and is used to process relatively limited number of samples. Additionally, lack of accuracy is an issue when the number of amoebae is low (< 100) in the hemacytometer chamber, and the amoebae tend to cluster.

Colorimetric methods based on the reduction of tetrazolium salts by metabolically active cells have been extensively used to measure cell viability and proliferation (Scudiero et al. 1988, Marshall et al. 1995, Berridge et al. 1996). The primary advantages of these colorimetric assays are their rapidity and precision, the avoidance of using radioisotopes, and the suitability for analyzing low numbers of cells (Hussain et al. 1993). Tetrazolium-based assays have successfully been used to assess the viability of several protozoans including the aquatic ciliates *Tetrahymena pyriformis* (Hegyesi & Csaba 1997) and *Anophryoides haemophila* (Cribb et al. 1999) and the amoebae *Naegleria fowleri* (Shibayama et al. 2003), *Entamoeba invadens* (Mukhopadhyay & Chaudhuri 1996), *Entamoeba histolytica* (Cedillo-Rivera et al. 1992, Bansal et al. 2004), and *Dictyostelium discoideum* (Bloomfield & Pears 2003).

Rapid detection and viability measurements of microorganism populations have been greatly enhanced by recent advances in flow cytometry (FCM) (Noble-Wang et al. 2004). The flow cytometer is defined as an instrument that allows acquisition of the optical characteristics of cells or particles (size, complexity, fluorescence) as they pass through a laser beam, in a single file (see Technical Sheet, Appendix 2), allowing for high numbers of cells (up to 10,000 cells/ min) to be analysed. The cell viability assay is only one of the numerous applications of flow cytometric techniques (Hawley & Hawley 2004). The use of fluorescent dyes allows for discrimination of viable and non

viable cells (Lloyd 1993). Propidium iodide (PI), one of the more common fluorescent dyes in FCM, has been widely used as an indicator of cell viability in different species (Garner & Johnson 1995, Soudant et al. 2005). The PI is a nucleic acid dye that permeates through membranes of damaged and dead cells only and emits a red fluorescence that can be detected by the FCM. The number of damaged or dead cells can thus be analyzed rapidly. By using fluorescent properties of dyes, FCM has been used to study and assess effects of biocides on several pathogenic amoeba species, including *Naegleria* and *Acanthamoeba* spp. (Muldrow et al. 1982, Khunkitti et al. 1997, Khunkitti et al. 1998, Borazjani et al. 2000, Connell et al. 2001, Noble-Wang et al. 2004).

In this study, the ability of both tetrazolium and flow cytometry-based assays to determine, rapidly and accurately, the viability of *N. pemaquidensis* after exposure to various treatments (sea water, freshwater, oxytetracycline, lasalocid and Virkon®S) was assessed. The treatments were used to set up assay parameters and not necessarily as potential therapeutic compounds *in vivo*. The suitability of *N. pemaquidensis* grown on rainbow trout gill cells as a source of parasites for screening assays was also carefully examined.

4.2 Materials and Methods

4.2.1 Culture of *Neoparamoeba pemaquidensis*

4.2.1.1 Stock Cultures of *Neoparamoeba pemaquidensis* and Rainbow Trout Gill Cells

Neoparamoeba pemaquidensis (ATCC 50172 isolated from coho salmon gills in 1988) were maintained on continuous sea water-based agar culture (ATCC medium 994, see Appendix 1) at ~ 20 °C in the dark. They were fed *Klebsiella pneumoniae* which were streaked onto the plate before addition of amoebae. Sub-culturing occurred every 4-7 d by cutting a cube of agar with growing amoeba and placing it face down on a fresh Petri dish seeded with bacteria.

The RTgill-W1 cells (ATCC CRL-2523) were maintained in 75 cm^2 polystyrene tissue culture flasks (Nunc) containing 10 mL Leibovitz medium (L-15, purchased from Sigma) supplemented with 10 % fetal bovine serum (FBS, Gibco) and 0.02 % of a L-glutamine / Penicillin / Streptomycin mix solution (Gibco). Flasks were kept in the dark at ~ 20 °C. Subculturing occurred when cells were confluent. Old medium was removed and cells were rinsed with EDTA. Cells were detached from the bottom of the flask by trypsinization. Fresh culture medium, L-15 with 10 % FBS (20 mL), was added to neutralize the trypsin. The cell suspension was dispensed to 2 new 75 cm^2 flasks (10 mL/ flask) (Lee et al. 2006).

4.2.1.2 Culture of *Neoparamoeba pemaquidensis* on RTgill-W1 Cells

Neoparamoeba pemaquidensis cultures (3-4 days old) were transferred from sea-water based solid media to confluent RTgill-W1 cell monolayers as described by Lee et al. (2006). The L-15 medium (330 mOsm/ kg) was progressively supplemented (within 72 h) with artificial 0.2 μm filtered sea water until adjusted to an osmolality compatible with survival of both gill cells and amoebae (close to 740 mOsm/ kg).

Amoebae were harvested by washing the agar several times with artificial 0.2 μm filter-sterilized sea water and were transferred to flasks containing RTgill-W1 cells at the adjusted osmolality. Control flasks containing either *N. pemaquidensis* or gill cells were also prepared. Amoeba attachment to the gill cells was monitored daily using an inverted microscope (Nikon Diaphot-TMD).

The cell monolayer was destroyed by the amoebae after ~ 96 h. Filter-sterilized, artificial sea water was then added to the flask. Any surviving RTgill-W1 cells were killed by the sudden increased salinity. Flasks were then gently rinsed twice with artificial sea water and amoebae were observed microscopically. Only flasks containing amoebae adhering to the bottom were used.

4.2.2 Treatments

Lasalocid and oxytetracycline were purchased from Sigma and Virkon®S was obtained from Pharmacal Research Laboratories. Drugs were prepared by dissolving directly in artificial sea water (oxytetracycline, Virkon®S) or in dimethyl sulfoxide (lasalocid) prior to dissolving in seawater. The final concentration of dimethyl sulfoxide

(DMSO) did not exceed 1 % in the amoeba suspensions. The drug solutions were filtered (0.2 μm) to remove debris or crystals. Final concentrations of lasalocid (0.1, 1, 10, and 100 mM), oxytetracycline (1, 10, 100 and 1,000 mM), and Virkon®S (10 ‰, 1 ‰, 0.2 ‰, 0.1 ‰) were prepared immediately prior to use. The freshwater used as a treatment in this study consisted of tapwater (water quality analysis in Appendix 3). All treatments were equilibrated at room temperature (~ 20°C) prior to use.

4.2.3 Viability Assays

4.2.3.1 Tetrazolium Salt-Based Viability Assay

The tetrazolium compound 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), also called Owen's agent, was purchased from Promega (Cell Titer $96_{Aqueous}$® One Solution Cell Proliferation Assay). The yellow MTS is reduced by metabolically active cells to a brown-coloured formazan product soluble in culture medium (filtered natural sea water) in the presence of an electron coupling agent PES (phenazine ethosulfate). The reagents are already supplied in one solution, ready to use after thawing.

Cytotoxicity assays were performed in 96 well-plates by addition of the reagent directly to treated culture wells, incubated for 4 h at room temperature (~ 20 °C), and the absorbance recorded at 490 nm with a plate reader (Spectromax ™ Microplate Reader, Molecular Devices). The absorbance of formazan is directly proportional to the number of metabolically active cells in culture.

Preliminary experiments were conducted in an attempt to establish the optimal parameters for the assay, i.e determinations of the appropriate concentrations of

amoebae and incubation periods, resulting in a linear relationship between number of protozoans and optical density (OD) throughout the assay. Amoebae cultured on rainbow trout gill cells were detached by firmly tapping the flasks against a rubber pad and harvested. The amoeba suspension was centrifuged for 5 min at 400 G. The supernatant was discarded and the pellet was resuspended in 0.2 μm filtered artificial sea water and centrifuged for 5 min at 400 G. The pellet was resuspended in artificial sea water. An aliquot of amoebae was stained with 0.5 % trypan blue (Sigma) (dissolved in 0.2 μm filtered artificial sea water) at 1:1 dilution to assess viability of amoebae and counted using a hemacytometer (Neubauer).

Amoebae were plated by serial dilution in a volume of 100 μL/ well into the 96-well plate (200,000, 100,000, 50,000, 25,000, 12,500, 6250, 1,562, and 781 amoebae/ well) using a repeating pipette at 20 °C. They were allowed to settle on to the bottom overnight (~ 12 h). Then, 20 μL of MTS-PES was added to each well using a repeating pipette. Amoebae were incubated for an additional 4 h prior to measurement of OD at 490 nm with a 96-well plate reader (Spectromax ™ Microplate Reader, Molecular Devices) at 20 °C. There were at least 3 replicate wells per amoeba concentration or incubation time.

The background OD was established by measuring sea water alone, sea water plus MTS, sea water plus the second supernatant, and sea water plus amoebae. Although potential growth of the protozoan would have been determined by increasing OD in the control wells, growth was not expected because *N. pemaquidensis* harvested from RTgill-W1 cells divide slowly in sea water as shown in Figure 2.3 (section 2.3.1, Chapter II).

4.2.3.2 Flow Cytometry-Based Viability Assay

Amoeba Suspension

Neoparamoeba pemaquidensis cultured on RTgill-W1cells in sea water were gently rinsed. They were detached by firmly tapping the flasks against a rubber pad, resuspended in 10 mL sea water, and transferred to a 15 mL plastic tube (Corning). An aliquot of amoebae was stained with 0.5 % trypan blue (dissolved in 0.2 μm filtered artificial seawater) (Sigma) at 1:1 dilution and counted using a hemacytometer (Neubauer) to assess viability of amoebae. The amoeba suspension was dispensed into 25 cm^2 cell culture plastic flasks (Corning) with a final volume of 5 mL/ flask and a concentration of 5 x 10^4 to 1 x 10^5 amoebae / mL. There were 4 replicate flasks per test material concentration including the no test material sea water control. After incubation overnight (~ 12 h) at room temperature (~ 20 °C), amoeba cultures were observed microscopically to assess attachment. Each flask was gently rinsed with sea water and filled with 5 mL fresh filtered sea water.

Sampling and Staining

Amoebae were suspended by tapping the flasks vigorously against a rubber pad. Detachment was monitored microscopically. Each flask was agitated manually and sampled by transferring 800 μL of the suspension to two different 5 mL polystyrene round bottom tubes (400 μL/ tube) (BD Falcon). Immediately after sampling, the first tube was analyzed by FCM to obtain the initial count of *N. pemaquidensis*. A 0.4 μL aliquot of PI (1 mg/ mL in filtered sterile water) (Sigma) was added to the second tube

and incubated for 30 min in the dark prior to FCM viability analysis (detection of fluorescence emitted by dead amoebae). Each flask was sampled at 0, 1, 7, and 24 h after the addition of the specified concentrations of test material. The complete experiment was repeated 4 times.

Flow Cytometry Analysis

Enumeration and measurement of fluorescence of amoebae were carried out using a Becton Dickinson® FACSCalibur™ flow cytometer connected to a computer using the operating system BD CellQuest™ Pro as data acquisition software. The optimized sheath fluid was purchased from BD Facs Flow. The principle of operation, the definition of the used terms, and the type of outputs from the FACSCalibur flow cytometer are described in Appendices 2, 4 and 5.

Density plots of live amoebae showed that *N. pemaquidensis* cells were homogeneously distributed in terms of forward scatter (FSC, proportional to the size) and side scatter (SSC, proportional to the cellular complexity) parameters and were easily defined by a red-highlighted region, R1 (Appendix 4). Parameters for analysis of FCM data were selected from observations on unstained viable and PI-stained senescent populations of amoebae. The PI-stained cells (~ 610 nm maximum emission wavelength) demonstrate more FL2 (orange-red fluorescence) than FL1 (green fluorescence) or FL3 (red fluorescence) characteristics via the FL2 detector of the flow cytometer (Appendix 5). Photomultiplier voltages settings were fixed throughout the experiment. The voltages were adjusted on the control panel for the FL1 and FL2 detectors so that viable populations produced histograms with a lower mean FL2

fluorescence (Histogram A, Appendix 5) than dead populations (Histogram B, Appendix 5). Viable amoebae excluded PI and produced low fluorescent signals from the FL1 and FL2 detectors. Dead amoebae produced a low fluorescent signal from the FL1 detector and a high fluorescent signal from the FL2 detector. Samples were run at a flow rate of 60 μL/ min. Only one measurement could be made on each sample. Count data were collected for 60 s (acquisition time) and eventually evaluated with FSC Express V2 software. Amoeba cell counts were analyzed for each treatment concentration using a two-way ANOVA with effects of test material treatment (versus control treatment, i.e. 0 concentration) and duration (1, 7, and 24 h versus 0 time), and their interaction. When differences in variances did not allow it, two-sample comparisons between test material and sea water control groups adjusted for multiple testing were used. Normality of the data was checked by visually assessing residual plots. When necessary, Log 10 and square root transformations were employed to ensure homogeneity. In all statistical analyses, significance was defined by $P < 0.05$. Statistical analyses were performed with Statgraphics Plus 5.0 software.

4.3 Results

4.3.1 Tetrazolium Salt-Based Assay

To determine the suitability of the MTS reagent for a compound screening methodology, we investigated the ability of various initial concentrations of *N. pemaquidensis* to reduce the tetrazolium salt to a measurable aqueous soluble formazan product. The amoebae were not able to reduce the salt during incubation; absorbance did not increase. Therefore, this method was deemed inappropriate in assessing the viability of *N. pemaquidensis*.

4.3.2 Flow Cytometry-Based Assay

4.3.2.1 Detection and Enumeration of Amoeba Populations

Amoeba populations could be distinguished from other particles and enumerated. Dead amoebae produced red fluorescence; PI staining occurred within 15 min. However, for technical convenience (time required to run each series of tubes), the incubation time was fixed at 30 min.

Preliminary experiments showed that cell counts in control samples decreased rapidly after a few minutes in the tubes. Rapid attachment of *N. pemaquidensis* to the walls of the plastic tube was suspected, justifying the use of two tubes: the first tube, assayed immediately after sample collection, allowed a total cell count (R1); after 30 min incubation with PI, viable cells adhered to the walls of the second tube whereas the dead fluorescent cells (M2) were in suspension and could be counted by FCM. The proportion of viable cells was calculated with the following formula:

$$[(R1-M2)/ R1*100)].$$

As growth in control flasks did not significantly change between 0 and 1 h (Appendix 6), they were only sampled at 0 h and not at 1 h to avoid any adverse effect due to frequent detachment. Also, to ensure that amoebae were attached when the treatment was added, treatment flasks were only sampled at 1 h and not 0 h. To improve the clarity of the presented work, only the most meaningful graphs are presented in this section. The others are placed in the Appendix Section (Appendices 7 to 9).

Freshwater (Figure 4.0, A) significantly reduced the number of live amoebae ($P < 0.05$) although adverse effects (i.e. detachment from the bottom and rounding up) were clearly observed at 6 h. At 24 h, amoeba cell counts in treatment flasks were ~ 4 times lower than in control flasks. Despite the lethal effects of freshwater on amoebae, as indicated by the large standard deviations, the number of treated amoebae was highly variable among flask replicates. Sea water controls and treatments were statistically different throughout the experiment.

Virkon®S dramatically reduced ($P < 0.01$) the number of amoebae at concentrations of 10 ‰ (Figure 4.0, B) and 1 ‰ (Appendix 7) compared to seawater controls. Amoeba counts in control flasks were not significantly different from treatment flasks at 0.2 ‰ and 0.1 ‰Virkon®S dilutions throughout the experiment (Appendix 7).

Oxytetracycline at the highest concentration (1,000 mM) significantly stimulated ($P < 0.05$) amoeba growth at 1 h (Figure 4.1, A). Lower concentrations of oxytetracycline (100, 10, and 1 mM) did not significantly alter viable cell counts compared to sea water controls (Appendix 8).

Lasalocid at the highest concentration (100 mM) significantly stimulated ($P < 0.05$) amoeba growth within 1 h (Figure 4.1, B). After this initial stimulation, growth

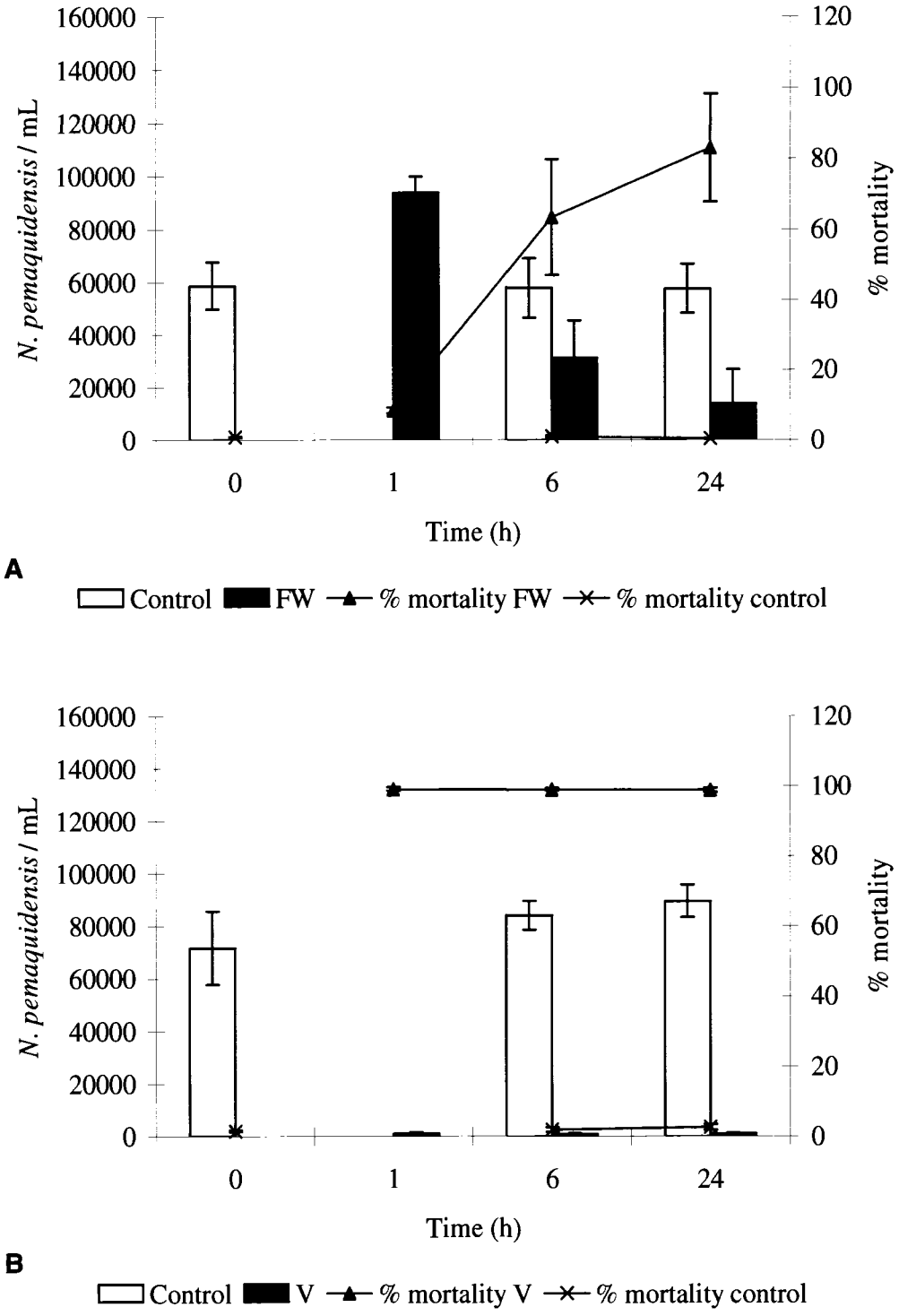
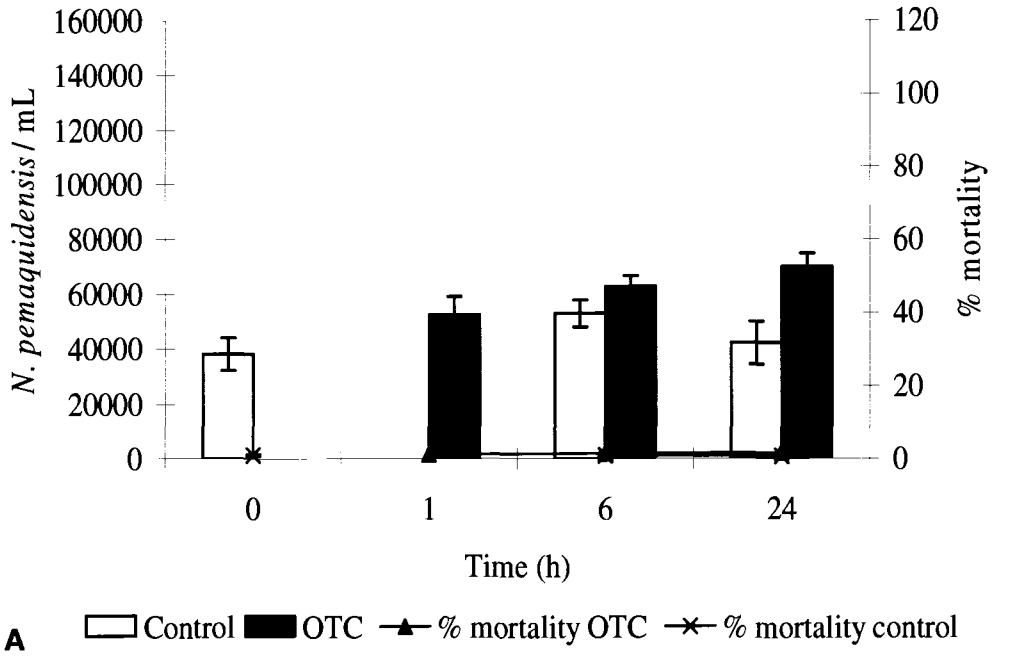
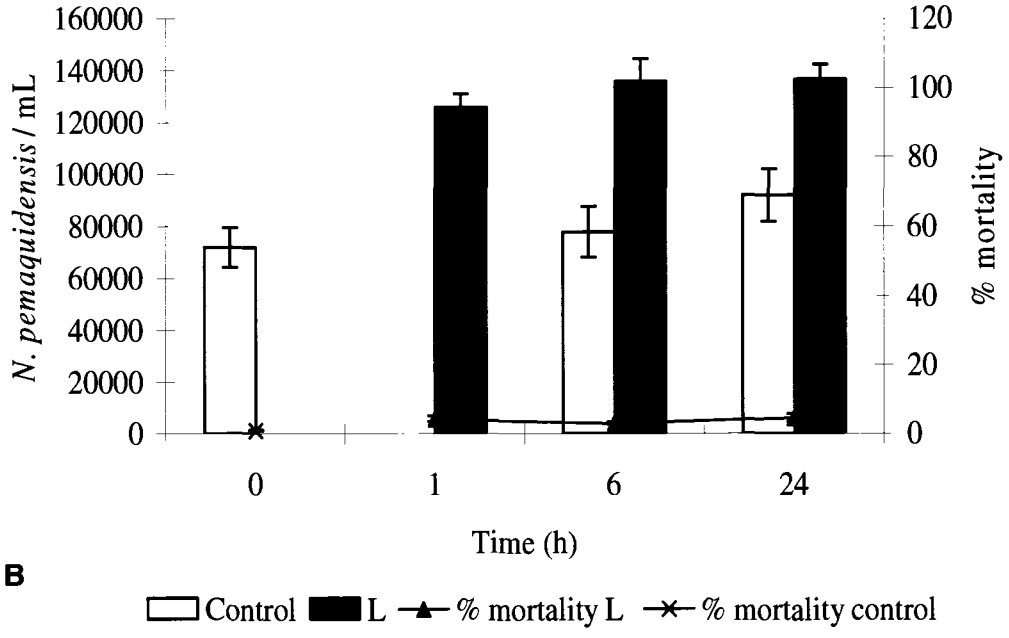


Figure 4.0: Total live amoeba cell counts after addition of (**A**) freshwater (FW) or (**B**) Virkon®S 10 ‰; (mean ± SD, n = 4).



A



B

Figure 4.1: Total live amoeba cell counts after addition of (**A**) oxytetracycline 1,000 mM (OTC) or (**B**) lasalocid 100 mM (L); (mean ± SD, n = 4).

was not observed in treatment flasks and cell counts remained stable. Lasalocid had no effect on viable cell counts at 10, 1 and 0.1 mM concentrations; control and treatment groups were not significantly different throughout the experiment (Appendix 9).

4.3.2.2 Effects of Treatments on Amoeba Viability

Freshwater (Figure 4.2, A) significantly reduced amoeba viability ($P < 0.05$), although adverse effects (i.e. detachment from the bottom and rounding up) were not demonstrated at 1h post treatment. Six h post treatment, viability had been reduced by 63 ± 16 %, while by 24 h, it had been further reduced by a total of 85 ± 15 %. Despite the demonstrated lethal action of freshwater on amoebae, the effect was highly variable among flask replicates as demonstrated by the large standard deviations.

Virkon®S killed amoeba efficiently at 10 ‰ (Figure 4.2, B) and 1 ‰ (Appendix 10) compared to seawater controls. Both concentrations caused ~ 99 % mortality within 1 h of exposure. The two other concentrations of Virkon®S, 0.2 ‰ and 0.1 ‰ (Appendix 10) dilutions, had no adverse effect on viability; treatment flasks were not significantly different from controls.

Oxytetracycline and lasalocid treatments did not influence amoeba survival, even at the highest concentration (1,000 mM and 100 mM, respectively) (Figure 4.3, A & B). Amoeba viability was consistently ~ 99 % and stayed stable throughout the experiment in control and treatment flasks. Amoeba viability at 24 h was not significantly different from amoeba viability at 0 h for all concentrations of oxytetracycline and lasalocid tested (Appendices 11 and 12).

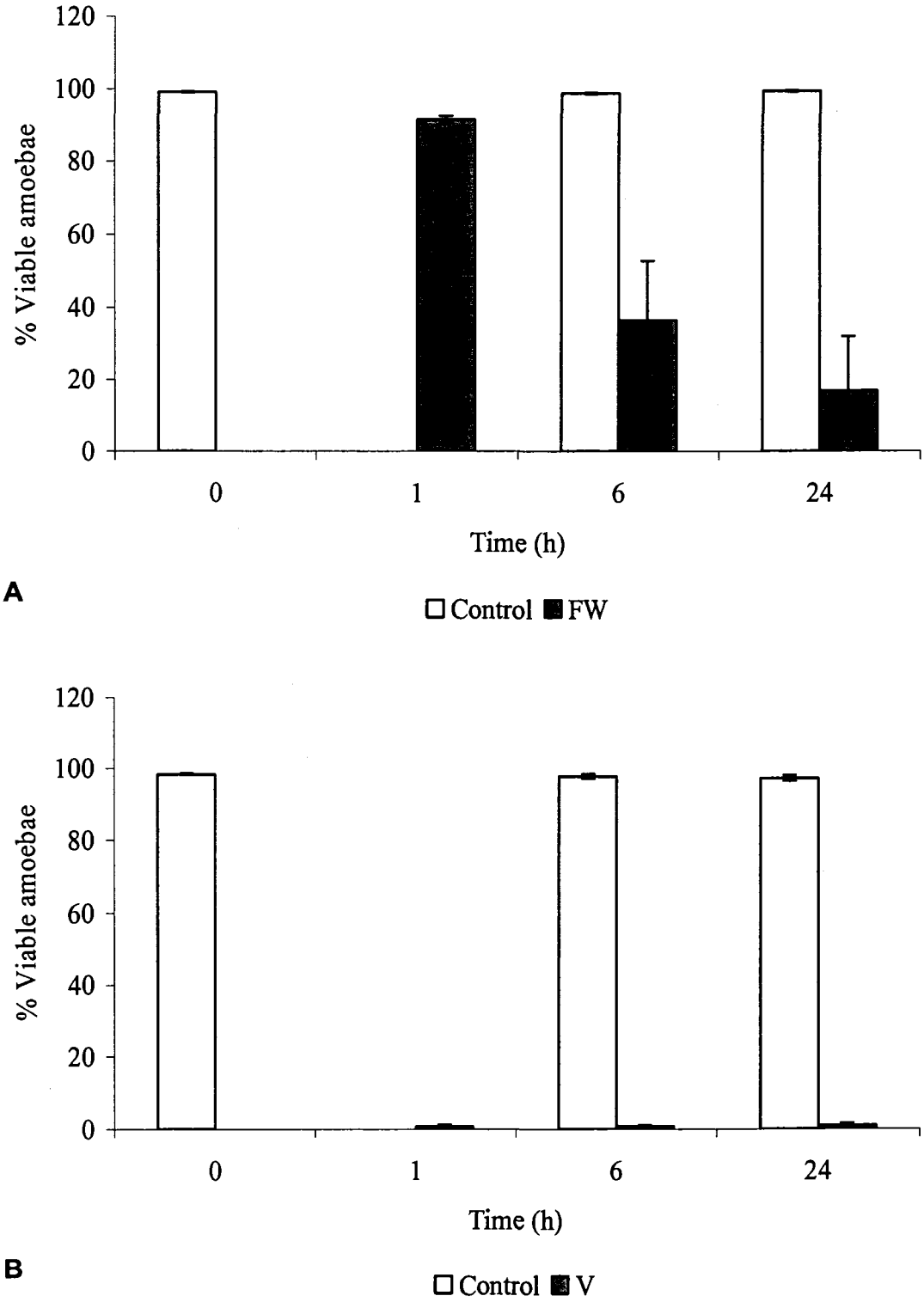


Figure 4.2: Percent viable amoebae of total amoeba cell counts after addition of (**A**) freshwater (**FW**) or (**B**) Virkon®S 10 ‰ (V); (mean ± SD, n = 4).

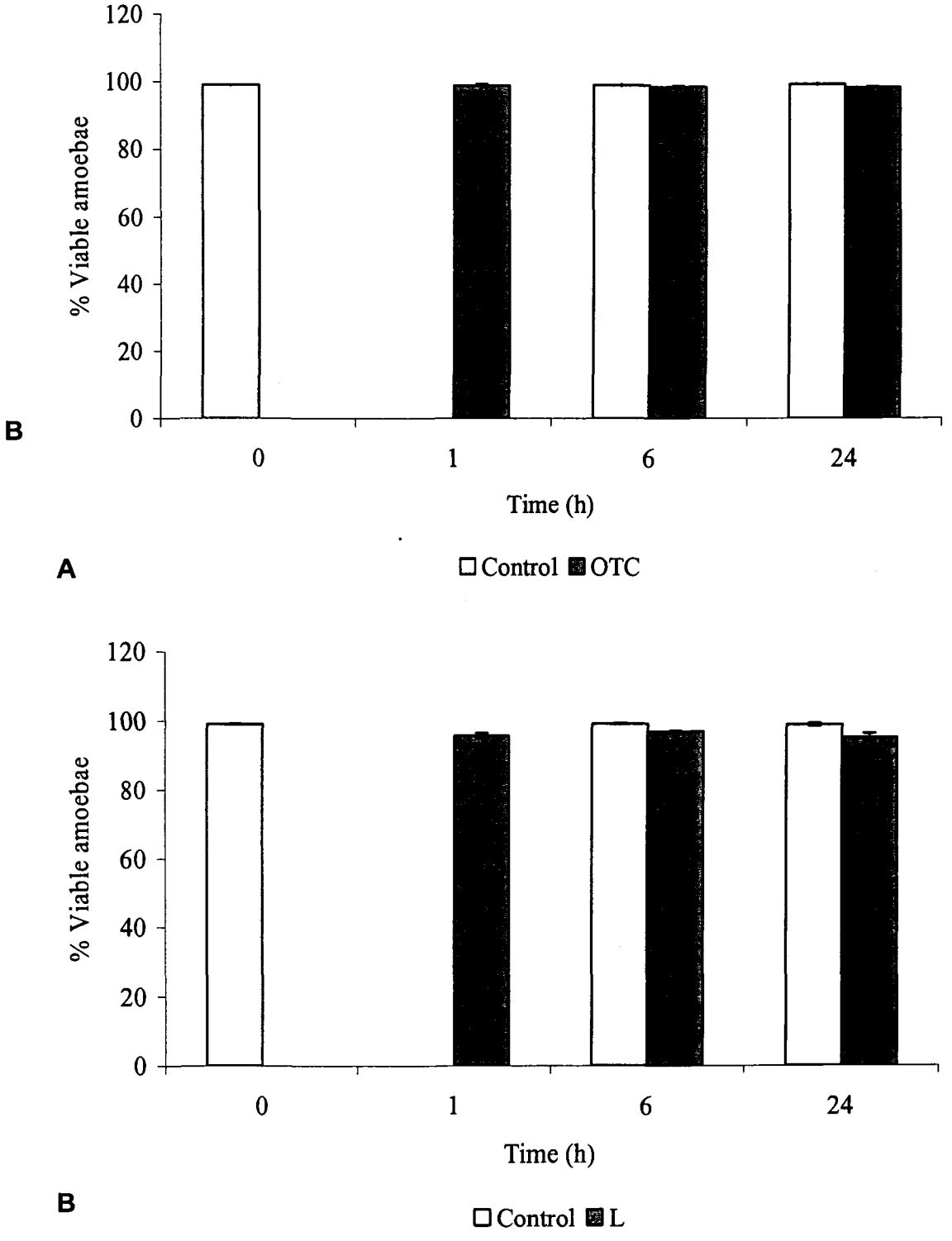


Figure 4.3: Percent viable amoebae of total amoeba cell counts after addition of **(A)** oxytetracycline 1,000 mM (OTC) or **(B)** lasalocid 100 mM (L); (mean ± SD, n= 4).

4.4 Discussion

The MTS was preferred over other tetrazolium salts as it provided more advantages in this type of study. First, it was available in kit form and thus was ready to add to the culture plates. Secondly, unlike nitro-blue tetrazolium (NBT) and [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), MTS does not require a volatile organic solvent to solubilise the formazan, and the culture plates can be returned to the incubator for further colour development. Finally, there are no washing or harvesting step that can cause cell loss and variability. The MTS was preferred to 3-bis-(2-methoxy-4-nitro- 5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), which also forms soluble formazan, because several studies demonstrated that the latter is unstable and therefore, not a reliable indicator of cell viability (Scudiero et al. 1988, Goodwin et al. 1995, Berridge et al. 1996).

In this study, MTS was not reduced by *N. pemaquidensis* harvested from RTgill-W1 cells, although the seeding density could reach 3 x 10^4 amoebae/ well. The ability of MTS to react with cells was then verified by adding it to RTgill-W1 cultures using similar methodology and conditions that were used for the amoebae (Section 4.2.3.1). As few as 4x10^3 RTgill-W1 cells were able to efficiently reduce MTS ($OD_{490\,nm} > 1$) during the 4 h incubation time (Appendices 13 and 14). The lack of response of *N. pemaquidensis* to the colorimetric assay was further investigated by using the non-aqueous soluble tetrazolium salt, MTT. The MTT-formazan forms purple crystals that can be observed by careful microscopic examination. The MTT was added to two amoeba cultures (ATCC 50172 and UA6 isolates) and to RTgill-W1 monolayers in 6-well culture plates. After 4 h incubation with this reagent, crystals could be observed

only in RTgill-W1 cell cultures (Appendix 15). Crystal formation was absent from amoeba cultures, even after 24 h incubation with MTT, suggesting that under the conditions of the assay, *N. pemaquidensis* was unable to reduce this tetrazolium salt or the MTS (Appendix 15).

The MTS assay does not directly measure cell viability; it only provides an estimation of cell viability via the measurement of NADH and NADPH dependent dehydrogenase activities in cells (Berridge et al. 1996). Although Mosmann (1983) showed the importance of mitochondrial dehydrogenases in the reduction of tetrazolium salt, it was eventually demonstrated that most cellular reduction occurred extra-mitochondrially (Berridge & Tan 1993, Marshall et al. 1995, Berridge et al. 1996). Several authors successfully established a colorimetric assay using tetrazolium salts (NBT and MTT) to study cytotoxicity and susceptibility to drugs of the amitochondrial amoeba, *Entamoeba histolytica* (Cedillo-Rivera et al. 1992, Mukhopahyay & Chaudhuri 1996). This reinforces the assumption that tetrazolium salts readily interact with electron transport systems, other than those from mitochondria, in amoebae.

As *N. pemaquidensis* cytoplasm contains ovoid or spherical mitochondria with tubular cristae (Dyková et al. 2000), the absence of signal after addition of tetrazolium compound observed in this study suggested that amoebae harvested from RTgill-W1 cells have low metabolic activity. This is in agreement with previous observations (Figure 2.3, A, Chapter II), indicating that *N. pemaquidensis* harvested from RTgill-W1 cells hardly grow when cultured in seawater.

Additionally, microscopic observation of culture flasks revealed that *N. pemaquidensis* were immobile and widely spread on the plastic bottom after destruction of the gill cell monolayer whereas they displayed movement and dividing activities immediately after addition to RT-gillW1 cultures.

As the growth curve of *N. pemaquidensis* ATCC 50172 harvested from agar ATCC 994 medium showed that *N. pemaquidensis* grow for the first 24 h following introduction into sea water (Figure 2.3, B, Chapter II), the amoebae were assayed with MTS according to the same protocol. Unfortunately, bacteria (*Klebsiella pneumoniae*) associated with amoebae rapidly proliferated and strongly reduced the tetrazolium compound. An additional assay was prepared with *N. pemaquidensis* CCAP 1560/ 5 (Table I, Chapter II) harvested from marine-based agar medium. This isolate does not require an additional bacterial food source, thus making the associated bacterial load quantitatively less important than ATCC 50172. However, this assay was fastidious to conduct for several reasons. First, it required large numbers of culture plates to yield sufficient numbers of test organims. Secondly, as the growth curves on agar media are unreliable (Appendix 16), amoeba harvest is decided by microscopic assessment of the cultures; this is subjective and does not give precise information on the growth phase. Finally, this assay required several centrifugation steps to lower the bacterial load, resulting in substantial loss of amoebae. After 8.5 h incubation with a maximal seeding density of 2×10^5 amoebae/ well, the absorbance reached only ~ 0.3 (OD) at 490 nm. Bacteria were observed in the well; however the extent of their contribution to the reduction of the tetrazolium salt could not be assessed.

Bracha & Mirelman (1984) suggested that virulence of *Entamoeba histolytica* may be related to its activity of oxido-reductive functions. Kumar et al. (1992) compared the ability of three strains of *E. histolytica* to reduce the tetrazolium salt NBT and observed that after 2 h incubation with NBT, the measured $OD_{572\ nm}$ of the *E. histolytica* isolates were respectively ~ 1.4 for the highly virulent IP 106 strain, ~ 0.4 for NIH: 200 strain (attenuated pathogenicity) and, ~ 1 for cholesterol-passaged NIH: 200 (NIH: 200 has a higher pathogenicity when cultured with cholesterol). The *N. pemaquidensis* harvested from RTgill-W1 cells in this study did not reduce the tetrazolium salt, consistent with its lack of pathogenicity.

While the use of tetrazolium-based viability assays has proven reliable, rapid and inexpensive for determining viability of *E. histolytica* (Cedillo-Rivera et al. 1992, Mukhopahyay & Chaudhuri 1996), the success of such an assay relies upon the use of axenic cultures of pathogenic amoebae harvested during their log phase (Cedillo-Rivera et al. 1992, Kumar et al. 1992, Mukhopahyay & Chaudhuri 1996). Unfortunately, these culture conditions are not presently achievable in paramoebiasis research (see Chapter II).

In contrast, our study demonstrated the feasibility of an *in vitro* flow cytometry-based method for counting and determining *N. pemaquidensis* viability following various treatments. Treatments for evaluation by the FCM assay were selected according to the following criteria: they are approved for use in terrestrial or aquatic farmed animals, they have different mechanisms of anti-protozoal activity, and they are inexpensive.

The primary treatment for AGD in marine finfish is a freshwater bath of 2-4 h duration. Powell & Clark (2003) showed that survival of *N. pemaquidensis* is prolonged in hard freshwater *in vitro* (> 50 mg $CaCO_3$/ L). Water is considered hard when the measured calcium carbonate equivalent is greater than 100 mg $CaCO_3$/ L. Analysis of the freshwater used in this study indicated a fairly high hardness (~ 150 mg $CaCO_3$/ L) (Appendix 3). Microscopically, amoebae exposed to freshwater adopted a spherical form because they were hypo-osmotically challenged. After 6 h in freshwater, 37 ± 16 % of the population of *N. pemaquidensis* were still alive. Despite the morphological change, amoebae could still adhere to the bottom of the plastic flask during the freshwater treatment, providing further support that amoebae survive freshwater treatments and could potentially reinfect fish.

This confirms the importance of calcium in amoeba survival. Calcium (Ca^{2+}) is essential as a second messenger for the control of various cell functions in eukaryotic cells, including contraction, secretion, and cell division (Moreno & Docampo 2003). Ravdin et al. (1985) suggested a role for Ca^{2+} in the adhesion and killing of target cells by *E. histolytica*. High levels of intracellular Ca^{2+} are critical for invasion of certain protozoan parasites such as *Toxoplasma gondii* (Carruthers & Sibley 1999). The Ca^{2+} regulation in parasitic protozoa differs in several aspects from the processes that occur in other eukaryotic cells (Moreno & Docampo 2003); this is an interesting target for further drug testing.

Virkon®S was first marketed for veterinary use as a surface disinfectant; it is an acidic mixture of peroxygen compounds (notably potassium monopersulphate) and organic acids that oxidize proteins and other components of cell protoplasm, resulting in

inhibition of enzyme systems and loss of cell wall integrity. The manufacturer recommends use of the product at a concentration of 10 ‰ for 10 min contact time. Hernández et al. (2000) showed that 10 ‰ Virkon®S effectively killed bacteria, yeasts and viruses but was not able to kill moulds and bacterial spores. Ares-Mazás et al. (1997) demonstrated that at higher concentration and longer contact time (60 ‰ for 2 hours), Virkon®S had some value for killing *Cryptosporidium parvum* oocysts, which are remarkably resistant to chemical disinfectants. As cysts have never been observed in *N. pemaquidensis*, resistance to Virkon®S was not expected and this was supported by our results. Given its biodegradability and low toxicity for users, Virkon®S appears as a suitable candidate for high-level disinfection in AGD-infected farms and facilities.

Oxytetracycline (OTC) is one of the most commonly used compounds in finfish aquaculture worldwide (Scott 1993, Burka et al. 1997). It is also the only chemotherapeutant approved for use in lobsters in North America. This compound has bacteriostatic action on cell growth by inhibiting protein synthesis (translation). The OTC has limited anti-protozoal action against amoebae and has been used as a treatment for amoebiasis. Seneca (1954) showed that *E. histolytica* was sensitive to this compound at concentrations ranging from 0.05 to 500 mg/ L *in vitro*. Bayer & Daniel (1987) found that the hemolymph of lobsters fed a medicated diet containing OTC can achieve concentrations of 1 mM (460 mg/ L). In sea bass, *Dicentrarchus labrax*, the concentration of OTC 24 h after *per os* administration (50 mg/ kg) was 2.6 mg/ L in plasma (Rigos et al. 2004). In this study, *in vitro* exposure to 1,000 mM oxytetracycline for 24 h did not cause any adverse effect on *N. pemaquidensis*, suggesting that this antibiotic would likely lack any amoebicidal effect in infected lobsters and fish.

Lasalocid is a divalent polyether ionophore antibiotic that binds and shields divalent metal ions within a hydrophobic matrix, and shuttles them across cell membranes, dramatically increasing ion transport. Lasalocid is approved for cattle and chickens with no required withdrawal period (Lindsay & Blagburn 1995). In this experiment, lasalocid had no adverse effects on *N. pemaquidensis* viability.

The disadvantages of FCM are associated with instrumentation costs, which are usually over $ 120,000 (CAN) for a typical laser-based flow cytometer (Noble-Wang et al. 2004). Another disadvantage is that skilled personnel are required to operate these complex instruments. In the current experiments, the FCM methodology overcame the technical difficulties encountered with the *in vitro* tetrazolium-based assay; the presence of bacteria and low cellular amoebic metabolic activity had no impact on enumeration and viability assessment of amoebae.

As FCM is one of the few technologies that can identify and analyze individual cells with respect to multiple parameters, it could be used in further research to measure quantitatively the morphological changes (size and complexity) undergone by treated amoebae throughout the experiment. Future screening assays of putative amoebicidal compounds using other isolates of *N. pemaquidensis* (Table I, Chapter II) could also be performed to determine and compare their respective susceptibility. Finally, it would be possible to test compounds on RTgill-W1 cells co-cultured with *N. pemaquidensis* to verify whether they selectively kill amoebae without deleterious effects on gill cells. The use of this *in vitro* model of AGD would therefore, help to eventually reduce the number of experimental animals used during *in vivo* testing.

In conclusion, this study showed that FCM is a suitable technique for viability assessment of *N. pemaquidensis*. However, the use of amoebae harvested from RTgill-W1 cells is not appropriate for screening compounds; the potential toxicity of certain drugs that act by inhibiting cell metabolism may be underestimated when added to amoebae in stationary phase. Therefore, efforts should concentrate on enhancing amoeba growth by improving the current artificial culture medium.

4.5 References

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V. GENERAL DISCUSSION

5.1 General Discussion

Neoparamoeba and *Paramoeba* spp. are the causative agents of paramoebiasis with important ecological and economical impacts in marine fisheries and aquaculture worldwide. These parasome-containing amoebae have been isolated from various hosts, and cause disease conditions ranging from systemic internal infection in invertebrates to gill surface colonization in finfish.

In the 1960's, recurrent epidemics in blue crabs, *Callinectes sapidus* occurred along the east coast from Connecticut to Florida (Sprague & Beckett 1966, Sawyer 1969). In this disease, caused by *Paramoeba permiciosa*, infected animals invariably died (Sprague & Beckett 1966, Newman & Ward 1973). The Atlantic coast of Nova Scotia, Canada, experienced two major epidemics of paramoebiasis in green sea urchins, *Strongylocentrotus droebachiensis*, between 1980 and 1983 (Miller & Colodey 1983, Scheibling & Stephenson 1984) and between 1993 and 1995 (Scheibling & Hennigar 1997). The disease, due to *Neoparamoeba pemaquidensis* (formerly *Paramoeba invadens,* Jones 1985), had a major impact on the ecology of this area (Miller 1985).

Neoparamoeba pemaquidensis is also the etiologic agent of amoebic gill disease, (AGD) in finfish. Outbreaks of AGD have been reported from most continents where intensive marine salmonid aquaculture is practiced (Munday 1986, Kent et al. 1988, Howard & Carson 1993). Additional to salmonid species, AGD is a recurrent problem in other cultured finfish species such as turbot, *Scophtalmus maximus* (Dyková et al. 1995). Amoebic gill disease continues to have a major impact, mainly in the Australian

salmon aquaculture industry with production losses estimated at 20 % (Munday et al. 2001).

Most recently, *N. pemaquidensis* has been implicated in a major mass mortality of American lobsters, *Homarus americanus*, during fall 1999 in Long Island Sound, USA (Mullen et al 2004, 2005). The 99 % reduction in landings (CTDEP 2000) caused the collapse of the local lobster fishery. As a sequel to the LIS lobster die-off, current research efforts continue to focus on the prevention of such catastrophes in other regions of north east America. The lobster fishery is an essential activity for the regional economy of the Atlantic provinces of Canada and coastal communities along the northeastern USA (Sackton 2004, Gardner & Pinfold 2006). Significant effort has also concentrated on accumulating information on all aspects of the biology of *N. pemaquidensis* with the objective of controlling the introduction of the pathogen in other regions. However, since the amoebae are amphizoic, they could potentially be found in any marine environment and thus making control program futile.

Understanding the biology of amphizoic amoebae such as *N. pemaquidensis* remains an enormous challenge. First, as with many protozoa, they are difficult to identify and their classification has undergone many changes (Adl et al. 2005). The distinctive morphological characteristics of the trophozoite and the presence of a parasome facilitate the identification of *Neoparamoeba* spp. However, since identification at the species and subspecies level is difficult, the creation of a *Neoparamoeba* DNA Database for identification of clinical and environmental samples would be very useful.

Secondly, the ecological role of amphizoic amoebae living freely in terrestrial and aquatic ecosystems is extremely complex. On one side, amoebae feed on and regulate bacterial populations (Weekers et al. 1993). On the other side, amoebae are essential in the dissemination, maintenance, survival and protection of bacteria in the environment (Robowtham 1986, Barker & Brown 1994). These potential associations have yet to be explored in paramoebiasis research and are important areas for further exploration.

Amphizoic amoebae have the potential to infect both immunocompromised and immunocompetent hosts (Schuster & Visvesvara 2004). Overall, diagnosis is difficult and prognosis is poor. Most infections caused by amphizoic amoebae are systemic and non-contagious (Newman & Ward 1973, Marciano-Cabral et al. 2000, Mullen et al. 2004). Interestingly, the agents of paramoebiasis are transmissible from host to host. The infection is systemic and consistently fatal in aquatic invertebrates (Sprague & Beckett 1966, Jones 1985, Mullen et al. 2004) whereas it remains localized to gills of finfish, which may survive the infection (Kent et al. 1988).

Knowledge on the factors that determine the virulence of the amphizoic amoeba *N. pemaquidensis* and its conversion from a free-living form or harmless gill epibiont to an aggressive invader is rather limited. The use of standardized *in vitro* cultivation methods for *N. pemaquidensis* should enable the study of many aspects of amoebic virulence in the absence of other microorganisms with which the trophozoites are usually associated.

Finally, there is no universal treatment for these amoebic infections (Schuster & Visvesvara 2004). In most cases, antimicrobial therapy is largely empirical and optimal

therapy, which is species-related, has to be determined. The current treatment for amoebic gill disease in marine fish farms consists of 2-4 h freshwater baths. With time, outbreaks of AGD have increased in severity and now occur throughout the year. This results from increased resistance of *N. pemaquidensis* to freshwater treatment and the lack of alternative treatment options. Presently, little information exists regarding the *in vitro* susceptibility of the pathogen to chemotherapeutants (Powell & Clark 2003); effective drugs against *N. pemaquidensis* are still unavailable.

The aim of this thesis was to investigate a newly established *in vitro* culture system, developed by Lee et al. (2006), consisting of growing *N. pemaquidensis* on rainbow trout gill cell lines (RTgill-W1). This methodology was first compared to the conventional sources of amoebae, i.e. culture on artificial medium and isolation from fish gills.

Culture of *N. pemaquidensis* isolates on artificial media is the most efficient way to maintain the collection of available *N. pemaquidensis* isolates and to produce pure amoebic cultures at low cost. However, these *in vitro* culture conditions do not efficiently yield sufficient numbers of amoebae for *in vitro* investigations and when harvested, they are heavily contaminated with bacteria. Finally, the major limitation to the use of *in vitro* culture on artificial media is that freshly isolated pathogenic *N. pemaquidensis* lose their virulence within approximately 34 days (Morrisson et al. 2005).

The maintenance and propagation of *N. pemaquidensis in vivo* is currently the only source of virulent isolates. The maintenance of the parasite in live animals is constraining and expensive. It also requires the isolation of *N. pemaquidensis* from the

gills. This is a delicate and time-consuming process, with extremely variable success. The major limitation to this *in vivo* method is the difficulty in isolating consistently pure cultures of *N. pemaquidensis* from gills.

The *in vitro* culture system established by Lee et al. (2006) provides high yield of *N. pemaquidensis* in a short time frame (~ 96 h). Although this cell culture system is not axenic, the bacterial load associated is reduced compared to that observed on artificial media. The observation that the amoeba isolate ATCC 50172, which is no longer virulent, is cytopathogenic toward the RTgill-W1 cells and exhibits morphological similarities with gill-isolated *N. pemaquidensis*, suggests that it may have regained its pathogenicity.

Therefore, this study explored the possibility that an avirulent *N. pemaquidensis* isolate grown on a gill-derived cell feeder layer may have recovered its initial pathogenicity towards susceptible hosts, i.e. Atlantic salmon and rainbow trout. Fish were challenged via immersion exposure with a highly concentrated inoculum of *N. pemaquidensis* harvested from RTgill-W1 cells. After 3 months post-exposure, fish exhibited neither gross nor histological evidence of AGD and *N. pemaquidensis* was not recovered from fish tissues.

This transmission study was unsuccessful in inducing AGD in Atlantic salmon and rainbow trout by exposure to *N. pemaquidensis* recovered from RTgill-W1 cell cultures. The issues raised by these results deserve further detailed studies, and many questions need to be answered. As level of pathogenicity is closely related to the strain of amoeba and environmental conditions, the next step would be to verify whether pathogenic gill-detached *N. pemaquidensis* may conserve their virulence when

immediately and continuously cultured on a gill-derived cell line. Concomitant to appropriate selection of a pathogenic isolate, the potential contribution of associated bacteria should also be carefully examined.

One of the advantages of the *in vitro* cell culture system established by Lee et al. (2006) is the rapid acquisition of a continuous and abundant supply of pure organisms, which may constitute a starting point in improving the drug screening process in paramoebiasis research (Powell & Clark 2003, Powell et al. 2003). Whether or not this source of the parasites is suitable for *in vitro* testing of therapeutants must be determined.

In vitro assays are useful and cost-effective for screening chemotherapeutants before testing on live animals. The aim of Chapter IV was to assess the ability of two different viability assays (tetrazolium salt reduction and flow cytometry-based (FCM) staining assays) to determine, rapidly and accurately, the viability of *N. pemaquidensis* harvested from RTgill-W1 cells. The test materials (freshwater, oxytetracycline, lasalocid, and Virkon®S) evaluated were used to establish assay parameters and not necessarily as potential therapeutic compounds *in vivo*.

Although the use of tetrazolium-based viability assays has proven reliable, rapid and inexpensive for determining viability of other amoebae (Cedillo-Rivera et al. 1992, Mukhopahyay & Chaudhuri 1996), it did not give satisfactory results in assessing *N. pemaquidensis* viability. However, the tetrazolium-based assay provided some insights on the low metabolic activity of the parasite harvested from RTgill-W1 cells.

In contrast, results from the FCM-based assay demonstrated the feasibility of this method for counting and assessing *N. pemaquidensis* viability after addition of various

treatments, including freshwater, oxytetracycline, lasalocid, and Virkon®S. However, amoebae harvested from RTgill-W1 cells may be inappropriate for further drug screening as they display a low metabolic activity, therefore possibly refractory to the potential toxicity of compounds that act by inhibiting cell metabolism.

In the immediate future, it is the author's opinion that research efforts should focus on improving the *in vitro* culture techniques, with respect to the maintenance of the collection of isolates, conservation of virulence, and improvement of the amoeba yield. First, since current sources of amoeba are not reliable, improvement of the cryopreservation methodology of clonal cultures of *N. pemaquidensis* is a priority. Secondly, studies aiming at enriching the culture medium and selecting appropriate bacterial food sources would also be of the utmost importance. Finally, attempts to culture various parasitic and environmental *N. pemaquidensis* isolates on feeder cell culture monolayers could facilitate understanding what triggers virulence. Success in the *in vitro* area would be subsequently followed by *in vivo* testing, the ultimate goal being the fulfillment of Koch's postulates.

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APPENDIX 1

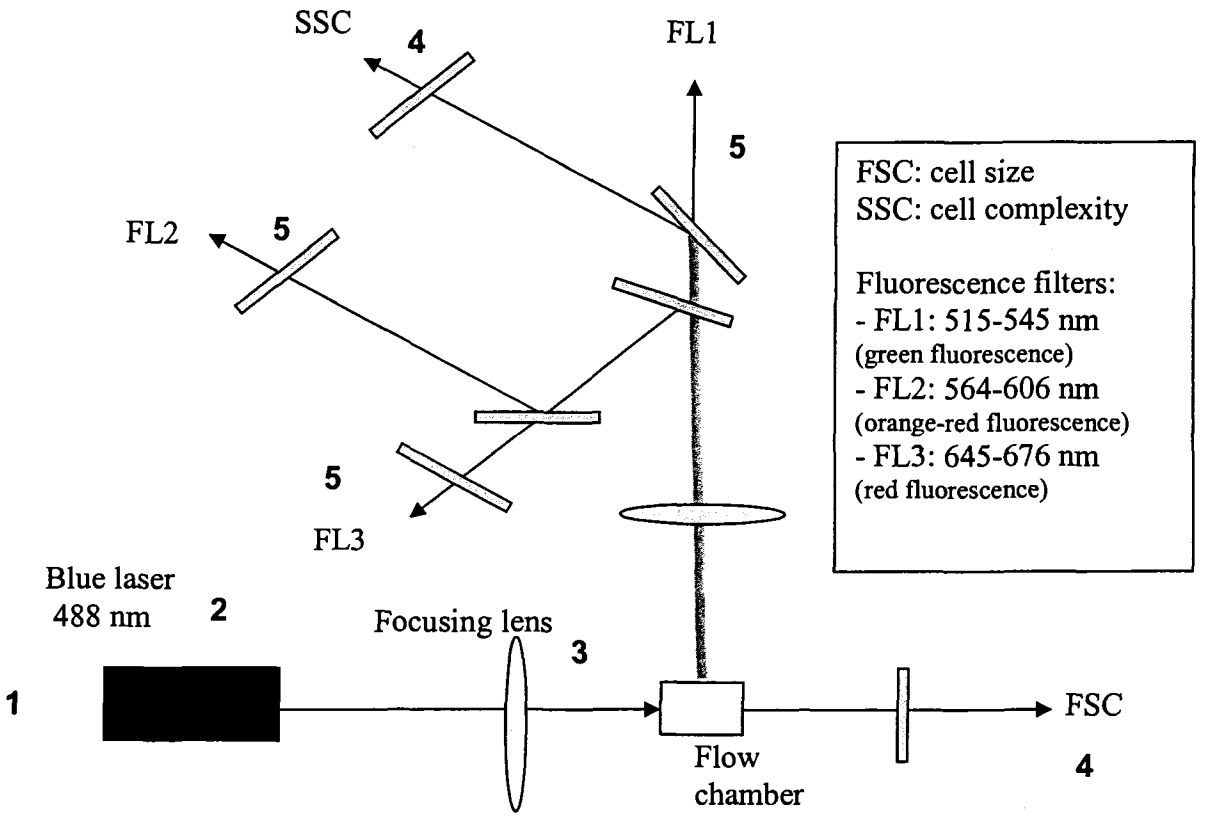
MEDIA	COMPONENTS	
ATCC 994	Malt extract	0.1 g
	Yeast extract (Oxoid L21)	0.1 g
	Agar (Difco 0140)	15 g
	Filtered artificial sea water*	1000 mL
MY75S	Malt extract	0.1 g
	Yeast extract (Oxoid L21)	0.1 g
	Agar (Difco 0140)	15 g
	Filtered artificial sea water*	750 mL
	Deionized water	250 mL
L1	Filtered artificial sea water*	950 mL
	$NaNO_3$	1 mL
	NaH_2PO_4	1 mL
	Metal	1 mL
	Vitamins	0.5 mL
	Agar (Difco 0140)	15 g

Recipes for culture media of marine amoeba

* Filtered (0.2 μm) artificial seawater (Instant Ocean®)

- Dissolve on a hot plate
- Autoclave at 121 °C for 25 min
- Bring to 50 °C before pouring the plates (approximately 18 mL per plate)
- Store the plates in the fridge (4 °C).
- Plates must be dried in the biosafety cabinet for several hours before use.

APPENDIX 2



Principle of the FACSCalibur ™ Optical system*

1: A sample (particles in suspension) is injected into a nozzle. The fluid is hydrodynamically compressed to a thin stream, forcing the particles to flow in a single file.
2: The stream of cells intercepts the beam of the laser and absorbs the laser light.
3: As particles flow through the beam, light is scattered; scattered light is either unmodified laser light or fluorescent light emitted from a fluorochrome that has been attached to the particle, such as propidium iodide (PI).
4: Unmodified scattered light is measured at low angle (FSC, forward angle light scatter), which gives a measurement of the particle size; scattered light is also measured as SSC (side angle scatter), which gives a measurement of the cytoplasmic complexity of the particle.
5: Emitted light is captured by lenses and filters to measure selectively emitted light of specific wavelength in a given detector.
6: Pulse signals sent by the detectors are converted into voltages and stored with computer software. PI-stained cells demonstrate more FL2 than FL1 characteristics via the FL2 detector of the flow cytometer.

*Becton Dickinson 1996

APPENDIX 3

P.E.I. Analytical Laboratories - Water Quality Test Report

440 University Avenue, Charlottetown, PE

Sample Number: 172690

Client Name Atlantic Vet College, U.P.E.I. **Project:** PRIVATE
Sample Location: 550 University Avenue.
Date Sampled: Oct 27, 2005
Date Received: Oct 28, 2005 **Water Type:** GW

Water Chemistry Results:

Method I.D.		Parameter:	Result	Units:	Detection Limit
WCL-04	*	pH (chem lab)	8.3	(pH units)	1
WCL-01	*	Alk Total	122.9	(mg/L)	8
WCL-01	*	Nitrate-N	3.2	(mg/L)	0.2
WCL-01	*	Chloride	14.7	(mg/L)	1
WCL-07		Barium	0.501	(mg/L)	0.01
WCL-07	*	Calcium	31.3	(mg/L)	0.01
WCL-07	*	Cadmium	< 0.005	(mg/L)	0.005
WCL-07	*	Chromium	< 0.05	(mg/L)	0.05
WCL-07	*	Copper	0.248	(mg/L)	0.02
WCL-07	*	Iron, Ext	< 0.1	(mg/L)	0.1
WCL-07	*	Potassium	1.54	(mg/L)	0.05
WCL-07	*	Magnesium	17.2	(mg/L)	0.01
WCL-07	*	Manganese	< 0.02	(mg/L)	0.02
WCL-07	*	Sodium	8.14	(mg/L)	0.1
WCL-07	*	Nickel	< 0.05	(mg/L)	0.05
WCL-07	*	Phosphorus	0.037	(mg/L)	0.02
WCL-07	*	Lead	< 0.002	(mg/L)	0.002
WCL-07	*	Sulfate	6.27	(mg/L)	1
WCL-07		Zinc	< 0.02	(mg/L)	0.02
WCL-07		Hardness (calc)	149	(mg/L)	0.1

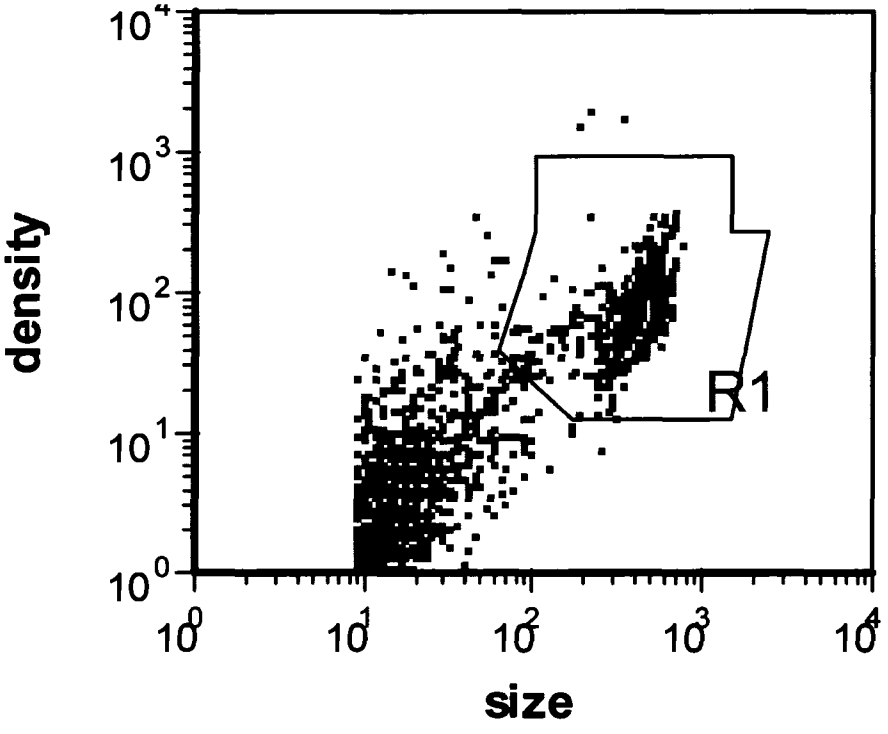
Approved By: Nancy Reeves Date: Nov 1, 2005

Date of Analysis available upon request.

MPN: most probable number; cfu/ 100 mls: colony forming units; mg/ L: milligrams per litre; nd: not detected; na: not analysed.
* Method accredited by Standards Council of Canada.

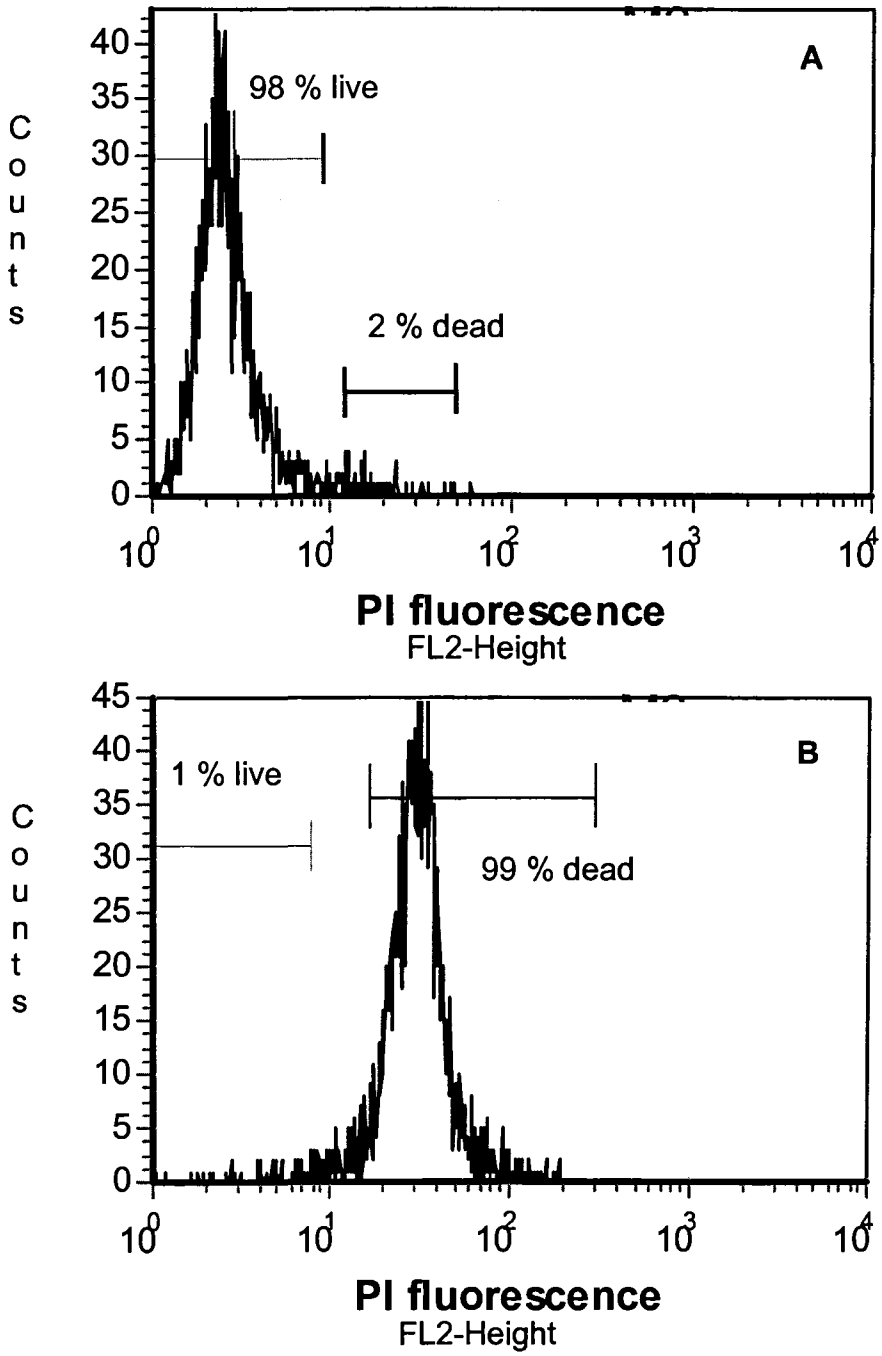
Charlottetown municipal water chemistry report (January 30, 2006)

APPENDIX 4



Forward scatter and side scatter density plot
Neoparamoeba pemaquidensis population appears in the red area, R1. The dots not included in R1 represent debris and bacteria present in the sample suspension.

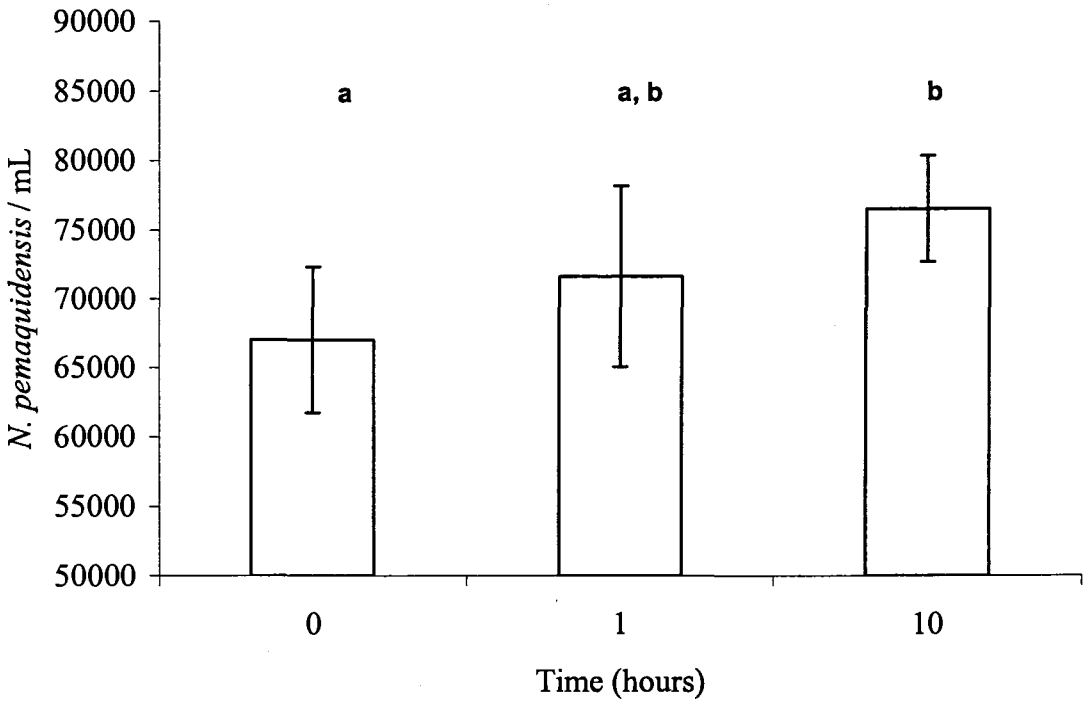
APPENDIX 5



Determination of FCM analysis parameters for viability assessment with PI

A) Viability assesment of amoebae before addition of the Virkon®S solution.
B) Viability assesment of amoebae 1 h after addition of the 10 ‰ Virkon®S solution.

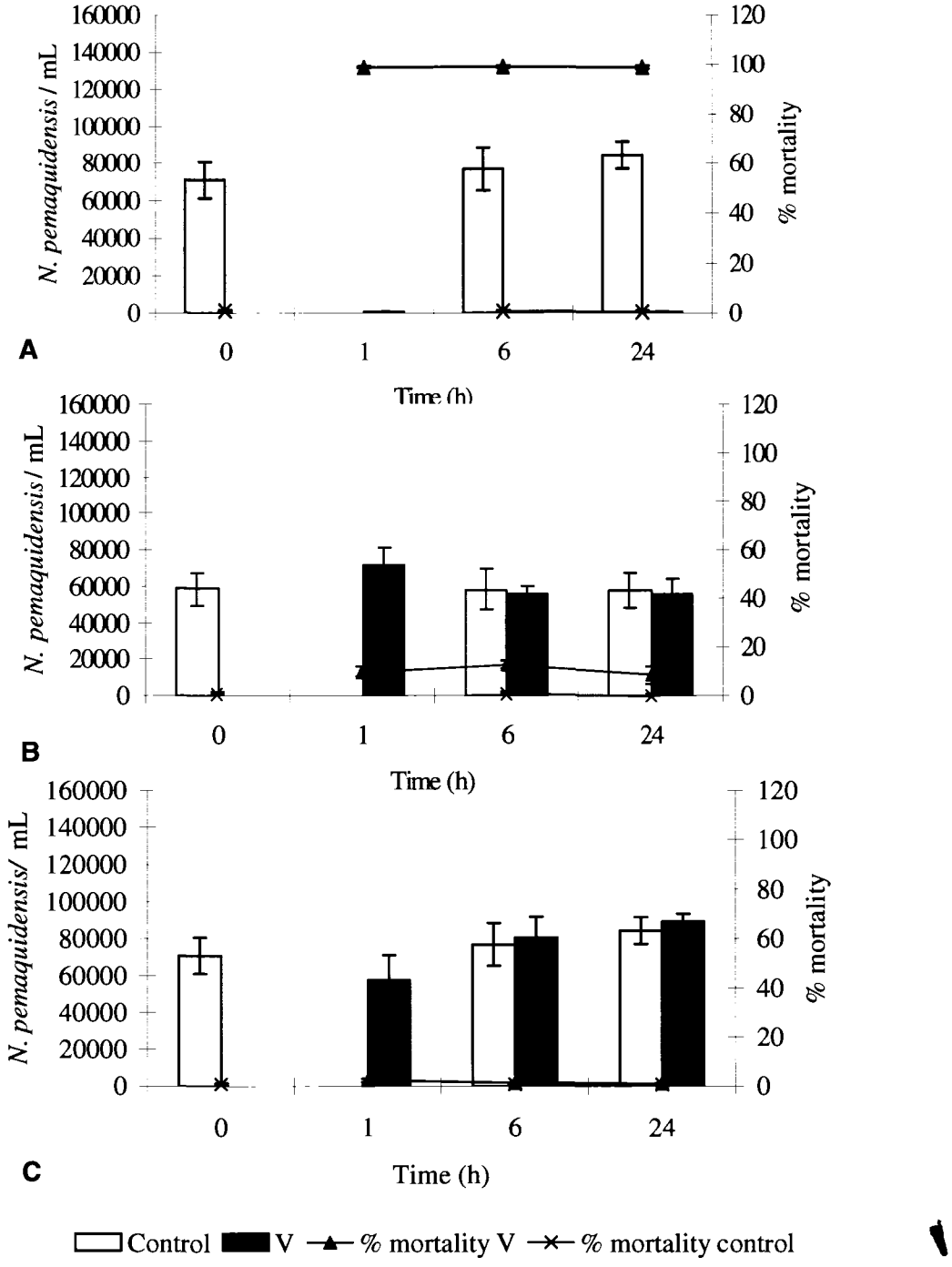
APPENDIX 6



Growth in sea water of *Neoparamoeba pemaquidensis* harvested from RTgill-W1 cells

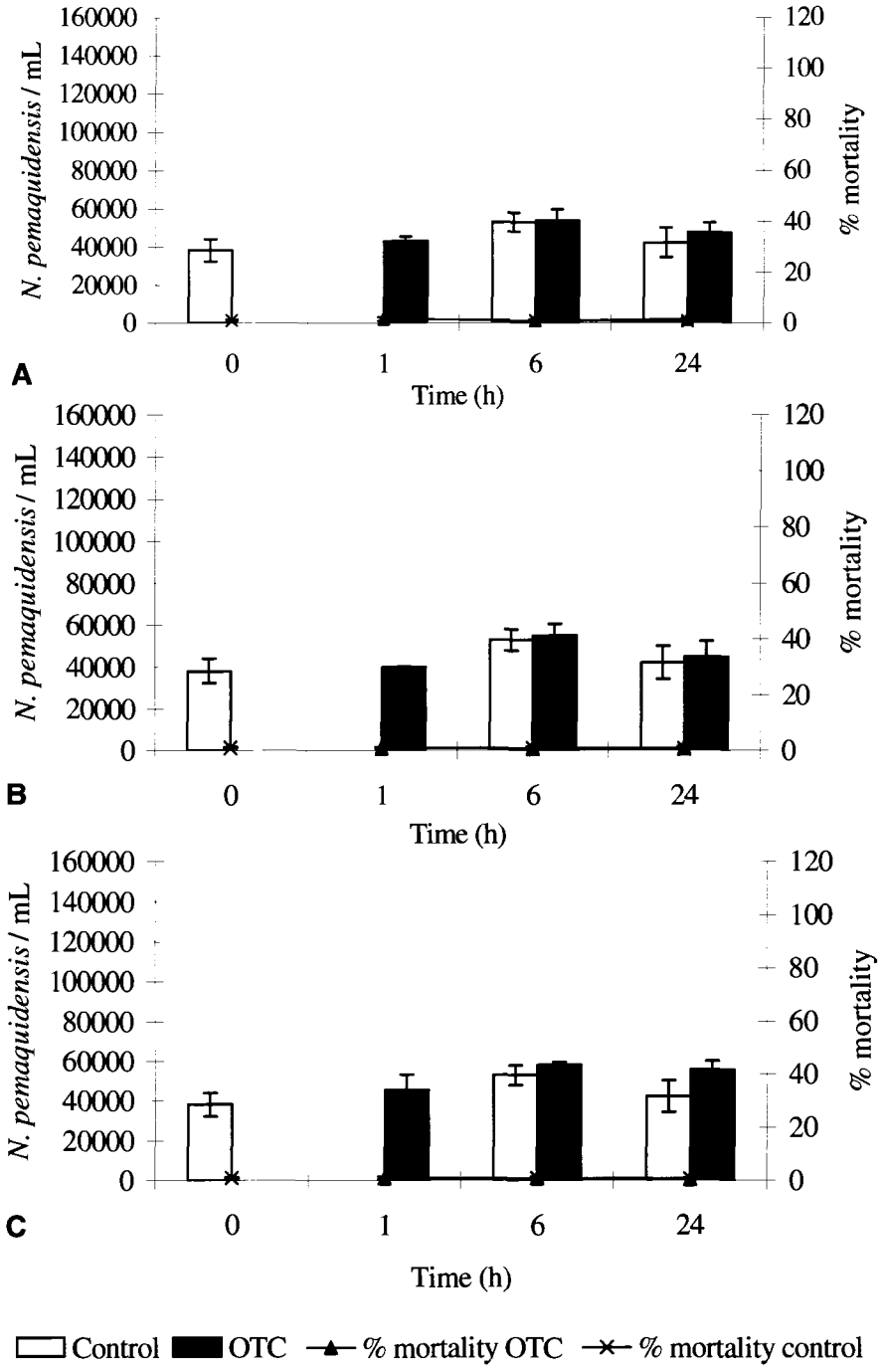
(mean ± SD, n = 4). Statistically significant differences are indicated by different superscript letters (a and b)

APPENDIX 7



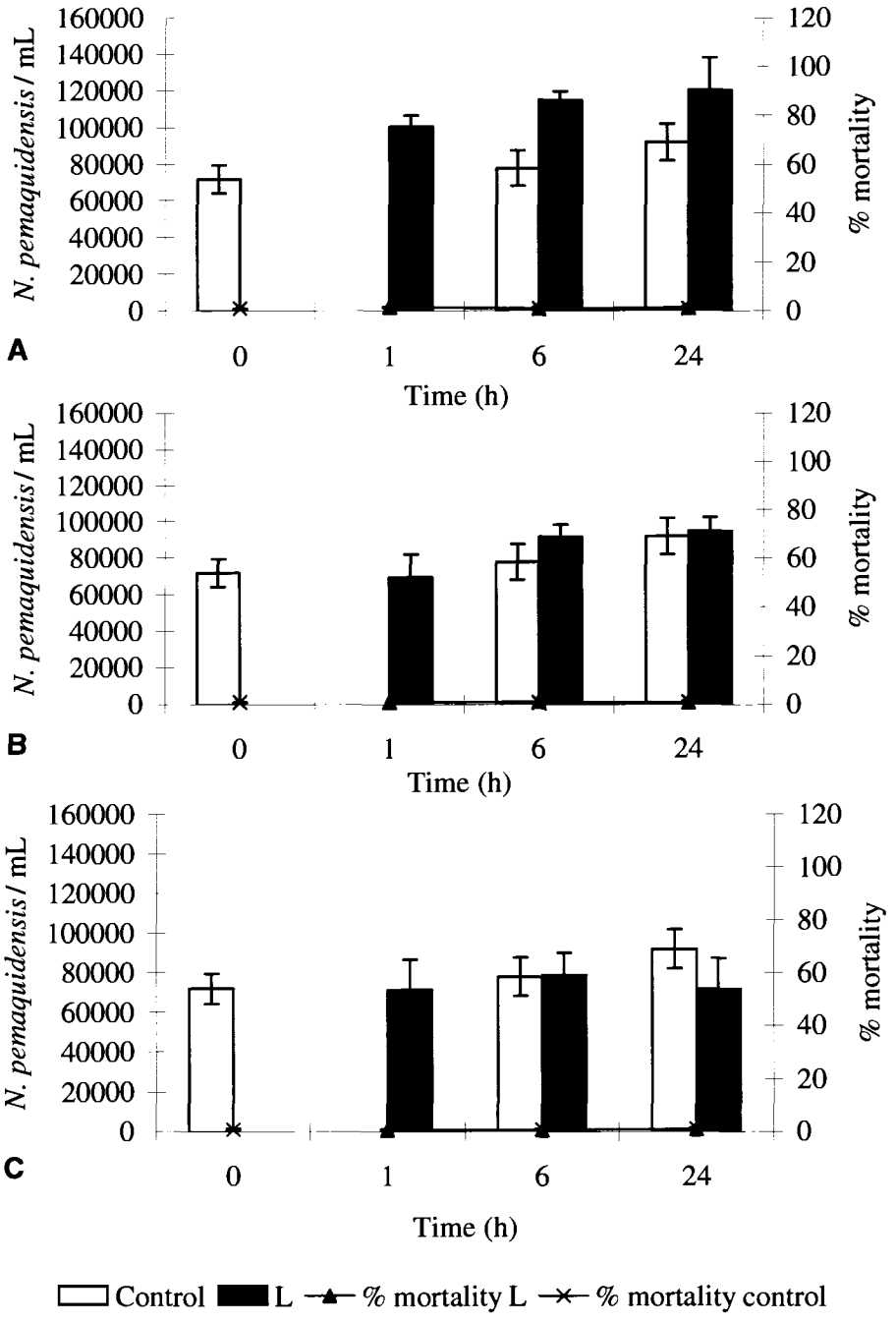
Live amoeba counts after addition of Virkon®S (V) at (A) 1 ‰, (B) 0.2 ‰ & (C) 0.1 ‰; (mean ± SD, n = 4)

APPENDIX 8



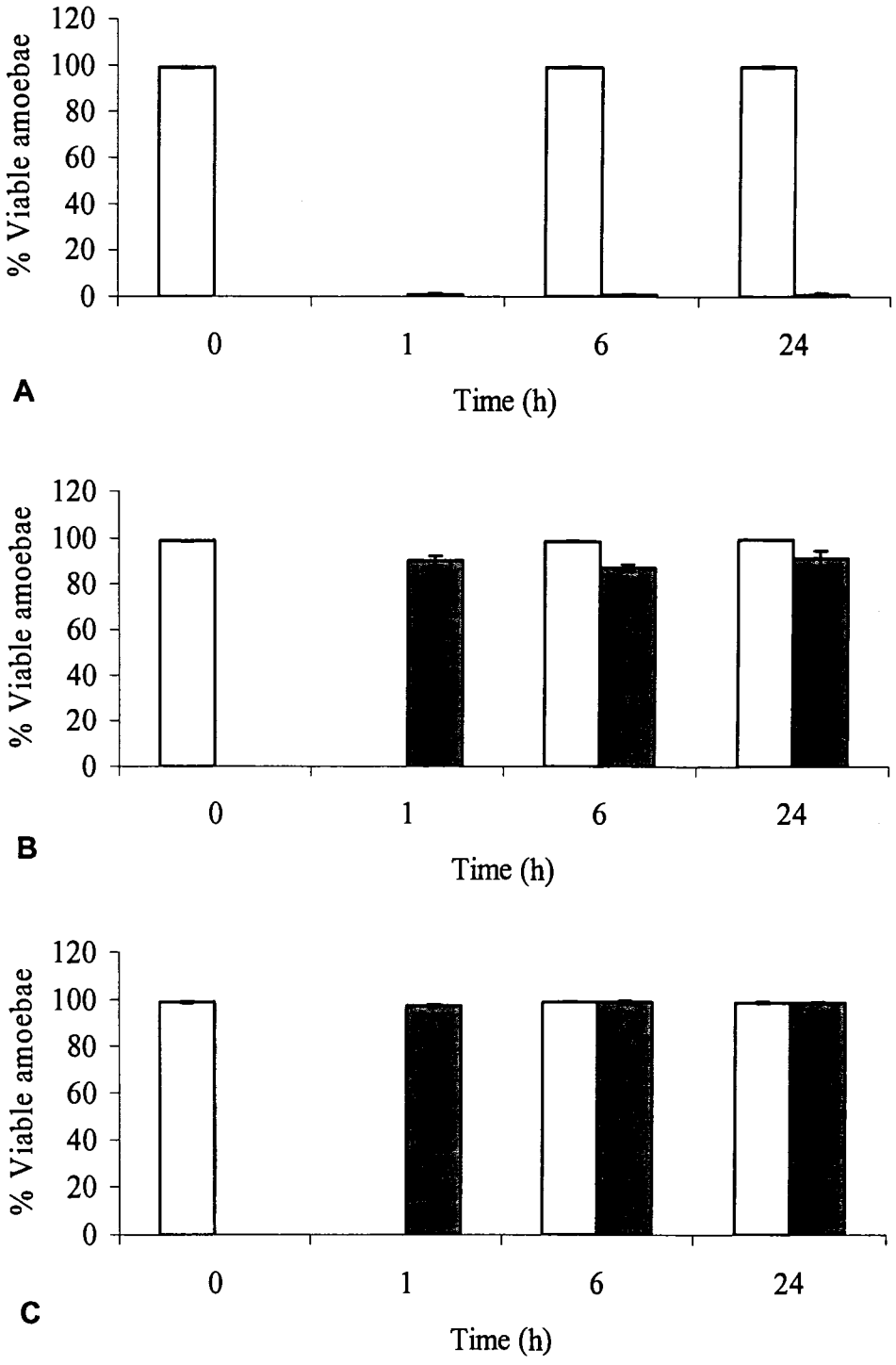
Live amoeba counts after addition of oxytetracycline (OTC) at (**A**) 100 mM, (**B**) 10 mM & (**C**) 1 mM; (mean ± SD, n = 4).

APPENDIX 9



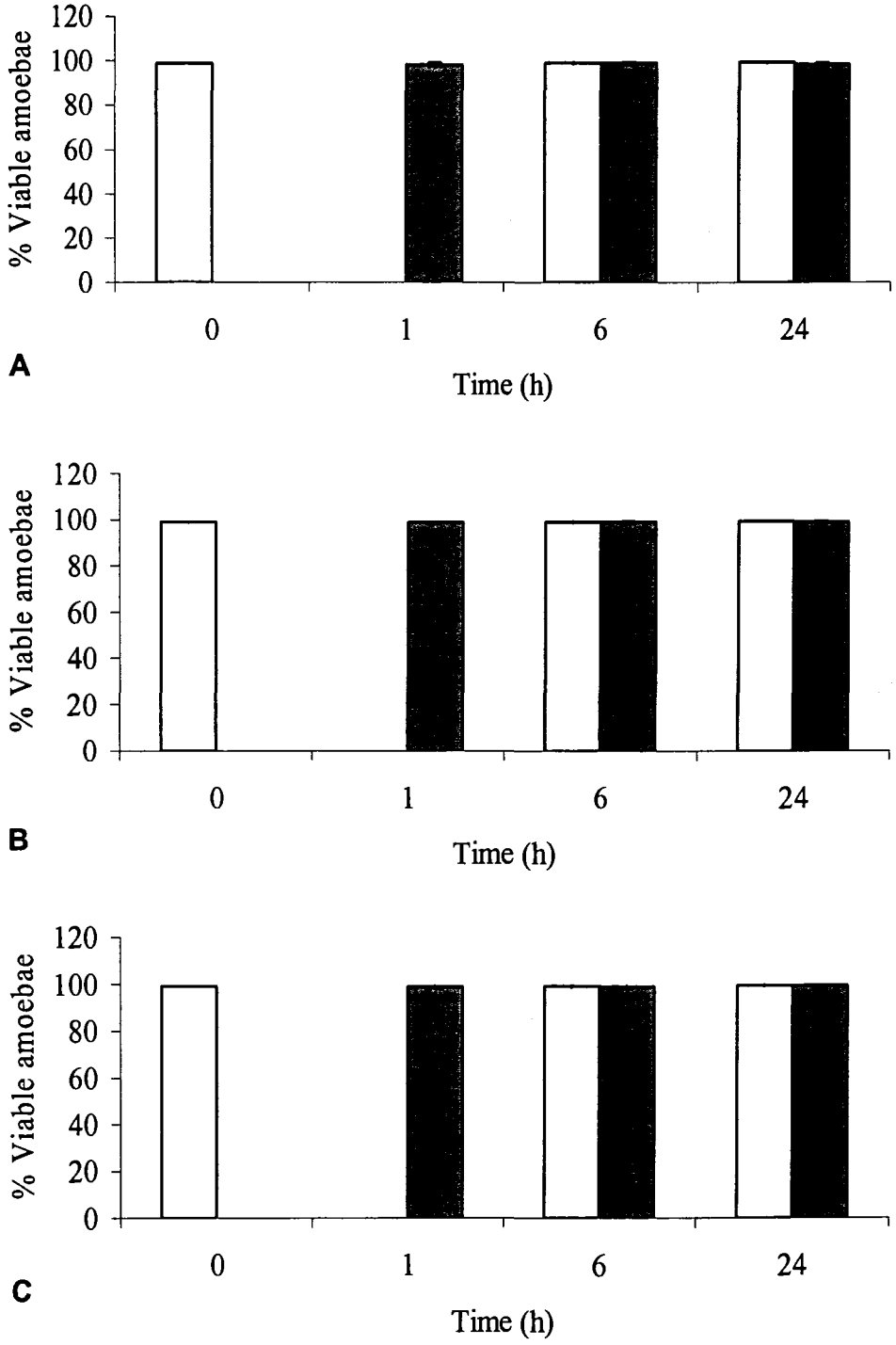
Live amoeba count after addition of lasalocid (L) at **(A)** 10 mM, **(B)** 1 mM, & **(C)** 0.1 mM; (mean ± SD, n = 4)

APPENDIX 10



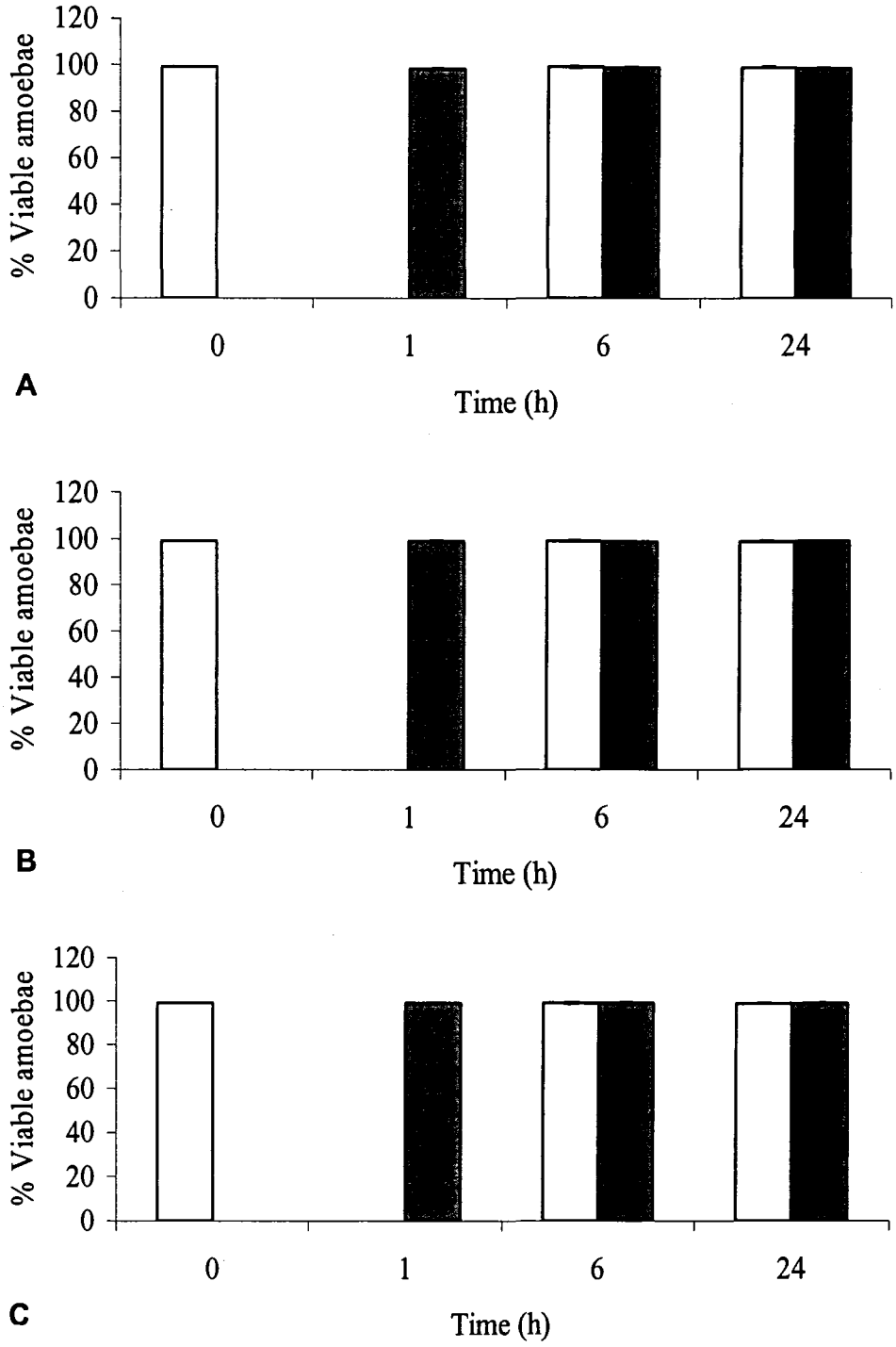
Amoeba viability after addition of Virkon®S (V) at (A) 1 ‰, (B) 0.2 ‰, & (C) 0.1 ‰; (mean ± SD, n = 4)

APPENDIX 11



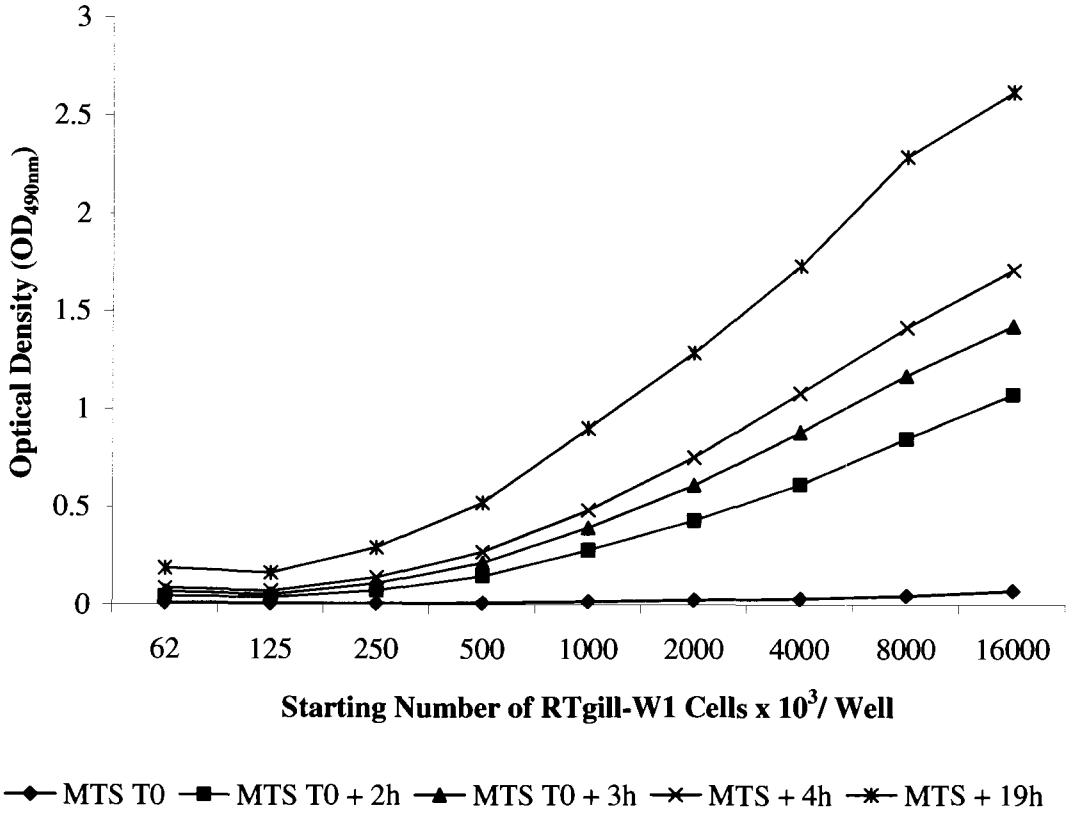
Amoeba viability after addition of oxytetracycline (OTC) at **(A)**100 mM, **(B)** 10 mM, & **(C)** 1 mM; (mean ± SD, n = 4)

APPENDIX 12



Amoeba viability after addition of lasalocid (L) at **(A)** 10 mM, **(B)** 1 mM, & **(C)** 0.1 mM; (mean ± SD, n = 4)

APPENDIX 13



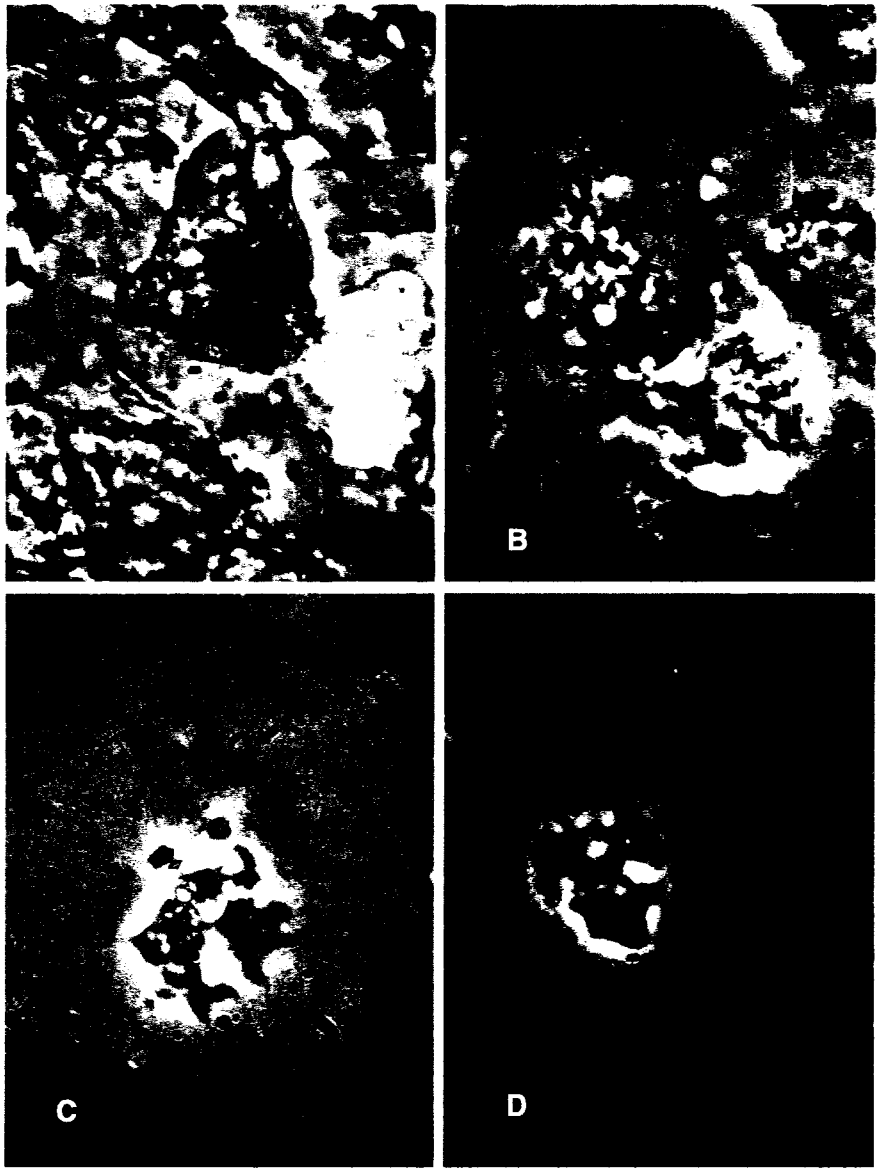
MTS-reducing activities of RTgill-W1 cells at various seeding densities and incubation times. Plotted values are averages of two replicate wells and raw data are presented in Appendix 14.

APPENDIX 14

Incubation time (h)	Starting number of RT-gill-W1cells x 10^3/ well	Replicate 1 OD_{490nm}	Replicate 2 OD_{490nm}	Average Replicates
0	62	0.009	0.010	0.009
0	125	0.008	0.007	0.007
0	250	0.007	0.006	0.006
0	500	0.009	0.011	0.010
0	1000	0.015	0.018	0.016
0	2000	0.025	0.024	0.024
0	4000	0.034	0.028	0.031
0	8000	0.046	0.048	0.047
0	16000	0.106	0.035	0.070
2	62	0.042	0.049	0.045
2	125	0.033	0.043	0.038
2	250	0.068	0.079	0.073
2	500	0.139	0.157	0.148
2	1000	0.274	0.289	0.281
2	2000	0.424	0.441	0.432
2	4000	0.604	0.626	0.615
2	8000	0.813	0.884	0.848
2	16000	1.163	0.981	1.072
3	62	0.060	0.072	0.066
3	125	0.048	0.061	0.054
3	250	0.100	0.118	0.109
3	500	0.206	0.226	0.216
3	1000	0.392	0.398	0.395
3	2000	0.594	0.628	0.611
3	4000	0.878	0.884	0.881
3	8000	1.129	1.210	1.169
3	16000	1.527	1.321	1.424
4	62	0.080	0.093	0.086
4	125	0.064	0.082	0.073
4	250	0.130	0.152	0.141
4	500	0.263	0.280	0.271
4	1000	0.480	0.488	0.484
4	2000	0.729	0.777	0.753
4	4000	1.079	1.081	1.080
4	8000	1.352	1.482	1.417
4	16000	1.823	1.596	1.709
19	62	0.178	0.200	0.189
19	125	0.149	0.182	0.165
19	250	0.270	0.317	0.293
19	500	0.496	0.549	0.522
19	1000	0.881	0.921	0.901
19	2000	1.244	1.329	1.286
19	4000	1.777	1.690	1.733
19	8000	2.261	2.312	2.286
19	16000	2.798	2.441	2.619

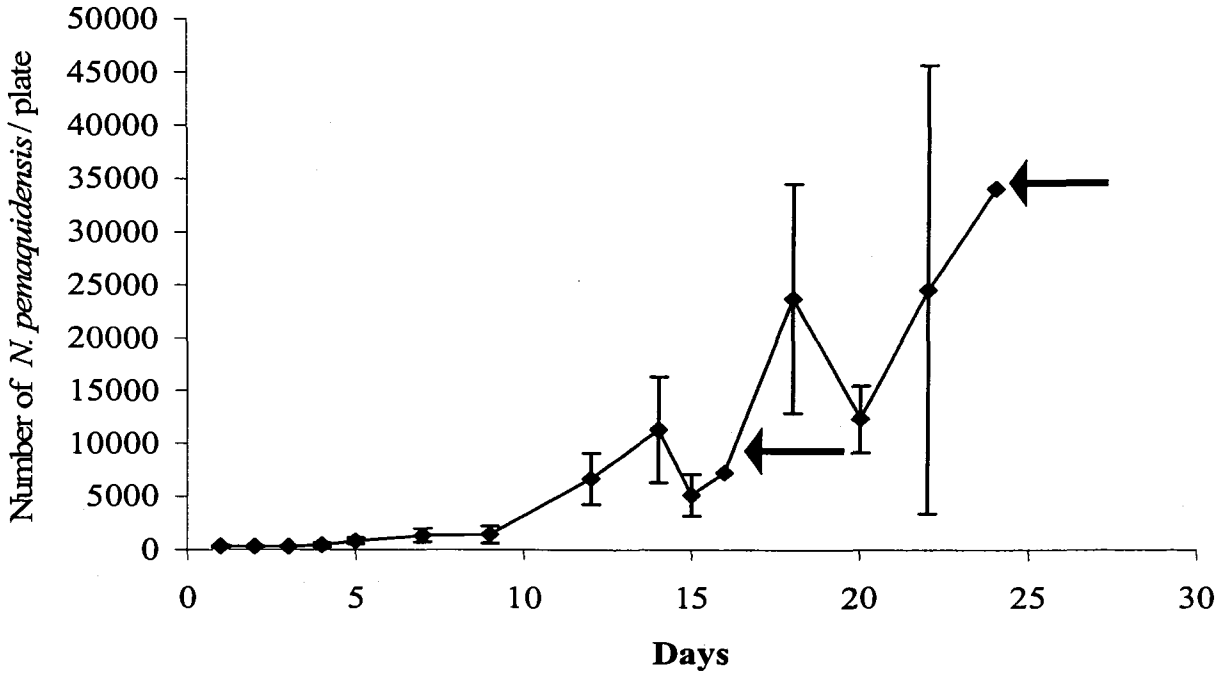
Raw data used to assess the MTS-reducing activities of RTgill-W1 cells

APPENDIX 15



Reduction of MTT in non soluble formazan product by RTgill-W1 cells and *Neoparamoeba pemaquidensis* (UA6 isolate). Purple crystals of formazan are visible within the RTgill-cell incubated 4 h with MTT (**A**) compared to RTgill-cells in sea water (**B**), bars =50 μm; Crystal formation was not observed in *N. pemaquidensis* after 24 h incubation with MTT (**C**) and there is no difference with *N. pemaquidensis* in sea water (**D**), bars =20 μm.

APPENDIX 16



Growth of *Neoparamoeba pemaquidensis* CCAP 1560/ 5 on MYS75S agar medium (mean ± SD, n = 3); two data points are the average of two counts only (arrows), which explains the absence of standard deviation.