

Role of bacteria in amoebic gill disease

by

Sridevi Embar-Gopinath

B.Sc., M.Sc., M.Phil.

Submitted in fulfilment of the requirements for the Degree of

Doctor of Philosophy

School of Aquaculture, University of Tasmania, Launceston,
Tasmania, Australia

December, 2006

Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university and, to the best of my knowledge contains no copy or paraphrase of material previously published or written by any other person, except where due reference is made in the text of the thesis.

Sridevi Embar-Gopinath

Authority of Access

This thesis may be made available for loan and limited copying in accordance with the Copyright Act 1968.

Sridevi Embar-Gopinath

Abstract

Neoparamoeba spp., are the causative agent of amoebic gill disease (AGD) in marine farmed Atlantic salmon, *Salmo salar* and AGD is the major problem faced by the salmonid industry in Tasmania. The only effective treatment to control AGD is freshwater bathing; however, complete removal of the parasite is not achieved and under favourable conditions AGD can reoccur within 10 days. Previous research on AGD suggests that gill bacteria might be one of the factors influencing colonisation of *Neoparamoeba* spp. onto Atlantic salmon gills. Therefore, the aim of this project was to investigate the role of salmonid gill bacteria in AGD.

To obtain good understanding of the bacterial populations present on Atlantic salmon gills, bacteria samples were collected twice during this study. Initially, bacteria were cultured from AGD-affected and unaffected fish both from laboratory and farm. These bacteria were characterised based on the colony morphology and biochemical profiles and identified using 16S rRNA gene-based approach. The bacteria from the genera *Winogradskyella* and *Staphylococcus* predominantly colonised the gills of AGD-positive Atlantic salmon; these bacteria were absent on AGD-negative samples. AGD-negative fish had a varied distribution of *Gammaproteobacteria* (*Shewanella baltica*, *Idiomarina* spp., *Pseudomonas* spp. and *Halomonas* sp.) and *Cellulophaga* spp., *Arthrobacter rhombi*, *Arenibacter troitsensis* and *Flavobacterium* sp..

Further, a series of *in vivo* experiments were conducted to study the influence of bacteria in AGD. The result showed an apparent involvement of *Winogradskyella* sp. in AGD. Fish experimentally infected with this bacterium developed significantly

more filaments (51% in chapter 5; 17% and 21% in chapter 6) with AGD lesions following challenge with *Neoparamoeba* spp. compared to the group that was exposed to *Neoparamoeba* spp. alone (16% in chapter 5 and 8% in chapter 6). Furthermore, this study also confirmed that *Neoparamoeba* spp. are able to infect salmonid gills and cause AGD with very low levels of culturable bacteria on the gills.

Based on the above results, it was important to verify whether bacteria such as *Winogradskyella* spp. that could exacerbate AGD conditions frequently colonise the gills of AGD-affected fish. Hence, a field-based study was conducted and bacteria samples were collected from marine farmed Atlantic salmon gills on five different occasions over a period of 102 days. The results showed that *Winogradskyella* species were not consistently present on AGD-affected fish gills. However, it was not possible to determine which bacteria will colonise the gills of AGD affected fish in a given period of time because the changes occurring within the gill bacterial population appeared to be complex and unpredictable and were influenced by several factors.

In conclusion, this study verified that the presence of bacteria on salmonid gills is not necessary to induce *Neoparamoeba* spp. infection; which further suggests that *Neoparamoeba* spp. is the primary causative agent of AGD. However, this study also demonstrated that the presence of *Winogradskyella* sp. on the gills during the process of AGD infection significantly increased AGD severity. This work forms the basis for future studies assessing the effect of bacteria and/or amoeba on fish and in investigating the role of other microorganisms that are recognized as potential factors influencing AGD.

Acknowledgements

I am grateful to my supervisor Dr Barbara Nowak, for all the guidance, encouragement and support during this project. Your enthusiasm, promptness and dutifulness has always inspired and motivated me to strive harder and to produce a better outcome each time.

I thank my associate supervisor Dr Phil Crosbie, who although took over this position mid-way has enthusiastically supported this project. Thanks for reviewing my manuscripts repeatedly and for providing useful comments. Our tearoom discussions/meetings always provided me with new ideas, approaches and directions.

I also like to thank Dr Jeremy Carson, Fish Health Unit, Dept, of Primary Industries and Water, Tasmania for providing initial training in Microbiology and for the feedback particularly on chapter 3. I appreciate Dr John Bowman, School of Agriculture, University of Tasmania, for helping me to learn PCR and sequencing and for providing useful comments on chapters 3 and 7.

I thank, Dr. Rick Butler, my previous associate supervisor, now at RSPCA, Tasmania, for all the support and help during the first year of my PhD. I also thank Dr Mark Powell (University of Tasmania) and his team for allowing me to collect gill bacteria samples during their field based trials and I would like to thank Tim Green for collecting these samples.

I thank my friends Thaila Subramanian, Rachna Mehta, Molly for their help with sampling during my laboratory experiments and Rebecca van Gelderen for providing me a hand during the mass production of culture media for bacteriology.

Thanks to Fisheries Society of British Isles, University of Tasmania and Aquafin CRC for funding my overseas travel to attend international conferences.

Also, thanks to Aquafin CRC for funding my Molecular biology training and for supporting this project. I acknowledge the Australian government for providing me the financial support through Australian Postgraduate Award.

Most of all, I like to thank my husband Murali, for his unconditional love and patience. Also, thanks for all the encouragement and motivation you have given me when I needed it most. Without your help and support, I would never have gotten this thesis completed.

Last but not least, I thank my mum, who continues to learn, grow and develop and who has been a source of encouragement and inspiration to me throughout my life. I am also grateful to my dad, my brother, my grandparents, my aunt and my cousin for their support and interest in my PhD.

Table of Contents

Declaration & Authority of Access	i
Abstract.....	ii
Acknowledgements.....	iv
Table of Contents.....	vi
List of Abbreviations	x
List of Figures and Tables	xi

Chapter 1. General Introduction..... 1

1.1	Tasmanian salmonid industry & amoebic gill disease (AGD)	2
1.2	<i>Neoparamoeba</i> spp., the aetiological agent of AGD	5
1.3	AGD pathology & diagnosis.....	6
1.4	AGD treatment & control	9
1.5	Factors influencing AGD.....	10
1.6	Interactions between bacteria & amoebae	10
1.7	Thesis aims & outline	12

Chapter 2. General materials & methods..... 14

2.1	Isolation of <i>Neoparamoeba</i> spp.	15
2.2	AGD diagnosis and assessment of severity	18
2.3	Bacteria isolation and morphological characterisation.....	20
2.4	Cryopreservation of bacteria.....	22
2.5	Biochemical characterisation of bacteria	22
2.6	Determination of standard curve.....	24
2.7	Cultivation of bacteria for experimental purpose	25
2.8	Extraction of bacterial DNA	26
2.9	Polymerase chain reaction (PCR)	26
2.10	Analysis of sequences	27
2.11	Sequence identification.....	27
2.12	Experimental tanks	28
2.13	Potassium permanganate treatment	29
2.14	Statistical analysis.....	29

Chapter 3. A culture-dependent 16S rRNA gene-based approach to identify gill bacteria associated with amoebic gill disease in Atlantic salmon 30

3.1	Abstract.....	31
3.2	Introduction.....	31
3.3	Materials and methods	33
3.3.1	Fish.....	33
3.3.2	Sample collection	34
3.3.3	Bacterial characterisation	34
3.3.4	Cluster analysis	35
3.3.5	16S rRNA analysis.....	35
3.4	Results.....	36
3.5	Discussion.....	44

Chapter 4. Use of potassium permanganate to disinfect Atlantic salmon gills 48

4.1	Abstract.....	49
4.2	Introduction.....	49
4.3	Material and methods.....	50
4.3.1	Experimental setup and treatment groups	50
4.3.2	KMnO ₄ treatment.....	51
4.3.3	<i>Neoparamoeba</i> spp. isolation and infection.....	52
4.3.4	Sampling procedures: Post-amoebae infection	52
4.3.5	Statistical analysis	52
4.4	Results.....	53
4.5	Discussion.....	57

Chapter 5. Influence of salmonid gill bacteria on development and severity of amoebic gill disease 59

5.1	Abstract.....	60
5.2	Introduction.....	61
5.3	Materials and methods	62

5.3.1	Experimental tanks and treatment groups	62
5.3.2.	<i>Neoparamoeba</i> spp. isolation.....	63
5.3.3	Bacteria cultivation	63
5.3.4	Bacteria characterisation	63
5.3.5	Potassium permanganate (KMnO ₄) treatment	64
5.3.6	Bacteria inoculation	64
5.3.7	Sampling procedures	65
5.3.8	Statistical analysis	65
5.4	Results.....	66
5.4.1	Histopathology	66
5.4.2	Bacterial recovery	67
5.5	Discussion.....	70

Chapter 6. Concentration effects of *Winogradskyella* sp. in the incidence and severity of amoebic gill disease..... 74

6.1	Abstract.....	75
6.2	Introduction.....	75
6.3	Materials and methods	76
6.3.1	Experimental setup.....	76
6.3.2	Treatment groups	77
6.3.3	<i>Neoparamoeba</i> spp. isolation.....	77
6.3.4	Bacteria cultivation and characterisation	77
6.3.5	Bacteria and amoebae exposure	78
6.3.6	Day 0 sampling	78
6.3.7	Day 8 sampling	79
6.3.8	<i>Winogradskyella</i> sp. retrieval.....	79
6.3.9	Statistical analysis	79
6.4	Results.....	80
6.4.1	Bacteriology	80
6.4.2	Assessment of AGD	80
6.5	Discussion.....	84

Chapter 7. Taxonomy of culturable bacteria isolated over time from marine farmed Atlantic salmon gills during a typical occurrence of amoebic gill disease	87
7.1 Abstract.....	88
7.2 Introduction.....	89
7.3 Materials and methods	90
7.3.1 Fish.....	90
7.3.2 Sample collection	91
7.3.3 Bacteriology	92
7.3.4 16S rRNA analysis.....	92
7.4 Results.....	93
7.4.1 <i>Gammaproteobacteria</i> and <i>Alphaproteobacteria</i>	93
7.4.2 <i>Cytophaga-Flavobacterium-Bacteroides</i> (CFB) phylum	94
7.4.3 <i>Firmicutes</i> and <i>Actinobacteria</i>	94
7.4.4 Comparison of pre (D49) and post (D49-PB) bathed samples ...	94
7.4.5 Comparison of post bath samples (D0-PB and D49-PB).....	95
7.5 Discussion.....	101
Chapter 8	107
General Discussion	108
References	119
Appendices	144
Appendix 1. Formulae of bacterial culture media	145
Appendix 2. Biochemical profiles of gill bacterial isolates (chapter 3)	147

List of Abbreviations

AGD	amoebic gill disease
ANOVA	analysis of variance
BLAST	basic local alignment search tool
CFU	colony forming unit
CRC	cooperative research centre
CFB	<i>Cytophaga Flavobacterium Bacteroides</i>
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra acetic acid
HOGG	head-on gilled and gutted
IFAT	indirect fluorescent antibody testing
kb	kilo base
PCR	polymerase chain reaction
KMnO₄	potassium permanganate
rRNA	ribosomal RNA
SDS	sodium dodecyl sulfate
SE	standard error
SS	sterile seawater
Tukey's HSD	Tukey's honestly significant difference

List of Figures and Tables

Chapter 1. General Introduction.....	1
Figure 1.1 View of Atlantic salmon sea cage farming in Tasmania	2
Figure 1.2 Annual HOGG production of Atlantic salmon in Tasmania	3
Figure 1.3 Map of Tasmania showing the location of AGD-affected (circles) and unaffected (square) salmon farms.....	4
Figure 1.4 Atlantic salmon gills with gross AGD lesions (arrows).....	7
Figure 1.5 Severely infected Atlantic salmon gill showing <i>Neoparamoeba</i> spp. (black arrows) on the surface of hyperplastic interlamellar units (HL), interlamellar vesicles (ILV) and infiltration of the central venous sinus with leucocytes (CVS).	7
 Chapter 2. General materials & methods.....	 14
Figure 2.1 Incubation of gill arches in Petri dishes.	17
Figure 2.2 Amoebae attached to the bottom of Petri dish 30-60 mins after incubation (a) and amoebae detached from the bottom of Petri dish 2-3 mins after addition of trypsin/EDTA (b).	17
Figure 2.3 A section of Atlantic salmon gills showing AGD-affected filaments (F1, F2 and F3) with hyperplastic lamellae (H) and vesicles (V) and unaffected filaments (F4 and F5) with healthy lamellae (arrows).....	19
Figure 2.4 Colony morphology and photographs of some of the bacterial colonies isolated from salmonids gills during the current study.....	21
Figure 2.5 API Zym (a) and API 50 CH (b) test results of <i>Winogradskyella</i> sp. strain AC1.	23
Figure 2.6 Standard curves for <i>Winogradskyella</i> sp. strain AC1 and <i>Staphylococcus</i> sp. strain AC8.....	24
Figure 2.7 Bacteria were evived using starter cultures (a) and after 24h were transferred into aerated 3 l carboy for large scale production (b).....	25
Figure 2.8 A single recirculating system showing three fish tanks and a sump	28

Chapter 3. A culture-dependent 16S rRNA gene-based approach to identify gill bacteria associated with amoebic gill disease in Atlantic salmon .. 30

Figures

Figure 3.1 Hierarchical comparison of the biochemical characteristics of bacterial strains isolated from AGD affected and unaffected fish gills.....**38**

Figure 3.2 16S rRNA gene-based phylogenetic tree indicating the location of bacteria phylotypes isolated from AGD affected and unaffected fish gills.....**41**

Tables

Table 3.1 16S rRNA gene phylotype identity of cluster representative of strains selected from AGD affected (groups A and B) and unaffected (group C) Atlantic salmon gills.....**39**

Table 3.2 Enzymatic profiles and carbohydrate fermentation reactions of the cluster exemplars that were selected for 16S rRNA analysis from AGD affected (groups A and B) and unaffected (group C) Atlantic salmon gills **42**

Chapter 4. Use of potassium permanganate to disinfect Atlantic salmon gills.....48

Figure 4.1 The mean \pm SE of the average number of bacterial colonies isolated from salmonid gills in experimental groups (group 1, no KMnO₄ exposure; group 2, exposed to KMnO₄; Control, no treatment). Separate analyses were done for each group and means with different letters above the bars represent groups that are statistically different. n = 6 fish per group.....**54**

Figure 4.2 The mean \pm SE of percentage of filaments with AGD lesions and number of infected lamellae within in each lesion in KMnO₄-treated and untreated groups. Separate analyses were done for each sampling day and the result was statistically insignificant. n = 6 fish per group per sampling day.....**55**

Figure 4.3 The mean \pm SE of average amoebae number on the entire gill surface in KMnO₄-treated and untreated groups. Separate analyses were done for each sampling day and means with similar alphabets above the bars represent groups that are statistically insignificant. n = 6 fish per group per sampling day.....**56**

Chapter 5. Influence of salmonid gill bacteria on development and severity of amoebic gill disease.....59

Figure 5.1 *Salmo salar*. The mean \pm SE of percentage of filaments with lesions and number of infected lamellae within in each lesion in experimental groups: (Group 1, *Neoparamoeba* spp.; Group 2, *Staphylococcus* sp. & *Neoparamoeba* spp.; Group 3, *Winogradskyella* sp. & *Neoparamoeba* spp.). Separate analyses were done for lesion percentage and lesion size (lamellar units). Different letters above error bars indicate groups are significantly different. n = 22 for all treatment groups.....**68**

Figure 5.2 *Salmo salar*. (A) Gram stained gill of AGD-infected salmon. *Winogradskyella* sp. (arrows) were present in large numbers on the surface of the lamellae (LM) and were free within the interlamellar space. Scale bar = 10 μ m. (B) Severely infected gill consisting of a continuous row of *Neoparamoeba* sp. (black arrows) on the surface of hyperplastic interlamellar units (H). Oedematous (O) gill tissue showing epithelial spongiosis (S), interlamellar vesicles (ilv) and infiltration of the central venous sinus with leucocytes (cvs). Scale bar = 100 μ m. (C) AGD affected gill showing interlamellar vesicles (ilv) containing *Neoparamoeba* sp. (arrows). Scale bar = 50 μ m.....**69**

Chapter 6. Concentration effects of *Winogradskyella* sp. in the incidence and severity of amoebic gill disease.....74

Figure 6.1 (a) Gill section of AGD-affected Atlantic salmon. *Winogradskyella* sp. (arrows) appears scattered in the interlamellar space adjacent to gill lamellae (L). Scale bar = 10 μ m. (b) Gill section showing severe AGD infection characterised by a row of amoebae (arrow heads) on the hyperplastic lamellar (H) surface and occurrence of interlamellar vesicles (ilv). Scale bar = 50 μ m.....**82**

Figure 6.2 The mean \pm SE of percentage of filaments with lesions and number of infected lamellae within in each lesion in treatment groups: (Group 1, *Neoparamoeba* sp.; Group 2, *Winogradskyella* sp. (10^8 cells l⁻¹) & *Neoparamoeba* sp.; Group 3, *Winogradskyella* sp. (10^{10} cells l⁻¹) & *Neoparamoeba* sp.). Separate analyses were done for lesion percentage and lesion size. Different letters above error bars indicate groups are significantly different.....**83**

Chapter 7. Taxonomy of culturable bacteria isolated over time from marine farmed Atlantic salmon gills during a typical occurrence of amoebic gill disease87

Figures

Figure 7.1 Composition of distinct bacterial species isolated from salmonid gills. Bars represent the abundances of different prokaryote groups (*Firmicutes*, *Actinobacteria*, *Alphaproteobacteria*, and *Gammaproteobacteria* *Cytophaga-Flavobacterium-Bacteroidetes* (CFB)) during various sampling times (D0-PB to D102).98

Figure 7.2 16S rRNA gene-based phylogenetic tree indicating the location of distinct bacterial phylotypes isolated on various sampling days (D0-PB to D102). Scale bar indicates maximum likelihood evolutionary distance. Numbers in parenthesis are GenBank accession numbers.....100

Tables

Table 7.1 A summary table showing sampling details, freshwater treatment time, seawater temperature (temp.), AGD severity and amoebae number.....96

Table 7.2 Mean (\pm SE) water quality parameters for freshwater bathing. ND = not determined 96

Table 7.3 Phylogenetic affiliations and frequency of isolates..... 97

Chapter 8. General Discussion.....107

Figure 8.1 Composition of gill bacteria cultured during this study from AGD-affected Atlantic salmon (chapters 3 and 7).....112

Chapter 1

General Introduction

1

General Introduction

1.1 Tasmanian salmonid industry & amoebic gill disease (AGD)



Figure 1.1 View of Atlantic salmon seacage farming in Tasmania

Photograph courtesy of M. Adams

Extensive salmonid aquaculture began in Tasmania in 1984 with an initial harvest of 20 tonnes of head-on, gilled and gutted (HOGG) Atlantic salmon (*Salmo salar*) during the summer of 1986/87 and by this year (2005/06) the annual production has attained 17,600 tonnes of HOGG (source: Tasmanian Salmonid Growers Association, www.tsga.com.au; figure 1.2). Tasmanian grown salmonids are of very high quality due to excellent growing conditions in pristine waters. In

terms of exports and imports, the domestic Australian market consumes most of the salmon produced in Tasmania (over 90%); however, there is also an important overseas market, particularly in Japan, Indonesia, Hong Kong and Singapore (Source: Dept. of Primary Industries and Water, Tasmania www.dpiwe.tas.gov.au).

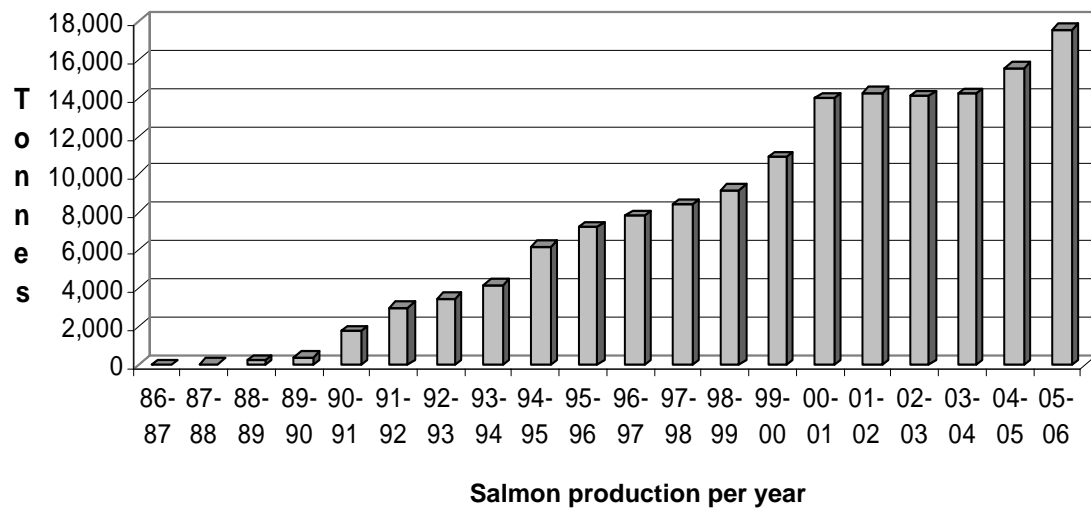


Figure 1.2 Annual HOGG production of Atlantic salmon in Tasmania (source: Tasmanian Salmonid Growers Association, www.tsga.com.au)

AGD is the only significant health problem confronted by the Tasmanian aquaculture industry. The first case of AGD was diagnosed in 1986 (Munday 1986). Corresponding to the increase in farming and production, the prevalence of AGD has also increased and expanded. As a result, farms where AGD was not a problem before, recently reported the occurrence of AGD. For instance, previously AGD was not recorded in the northern part of Tasmania i.e. in Tamar River but lately this farm

also reported the occurrence of AGD. Currently, Tasmania can be divided into two zones with regard to the AGD status, one where AGD is present (farms located in D'Entrecasteaux Channel, Tamar River and Huon River) and one that is free of AGD (Macquarie Harbour; Figure 1.3). Several environmental factors could be responsible for this difference including tidal range and salinity (Douglas-Helders et al. 2005a).

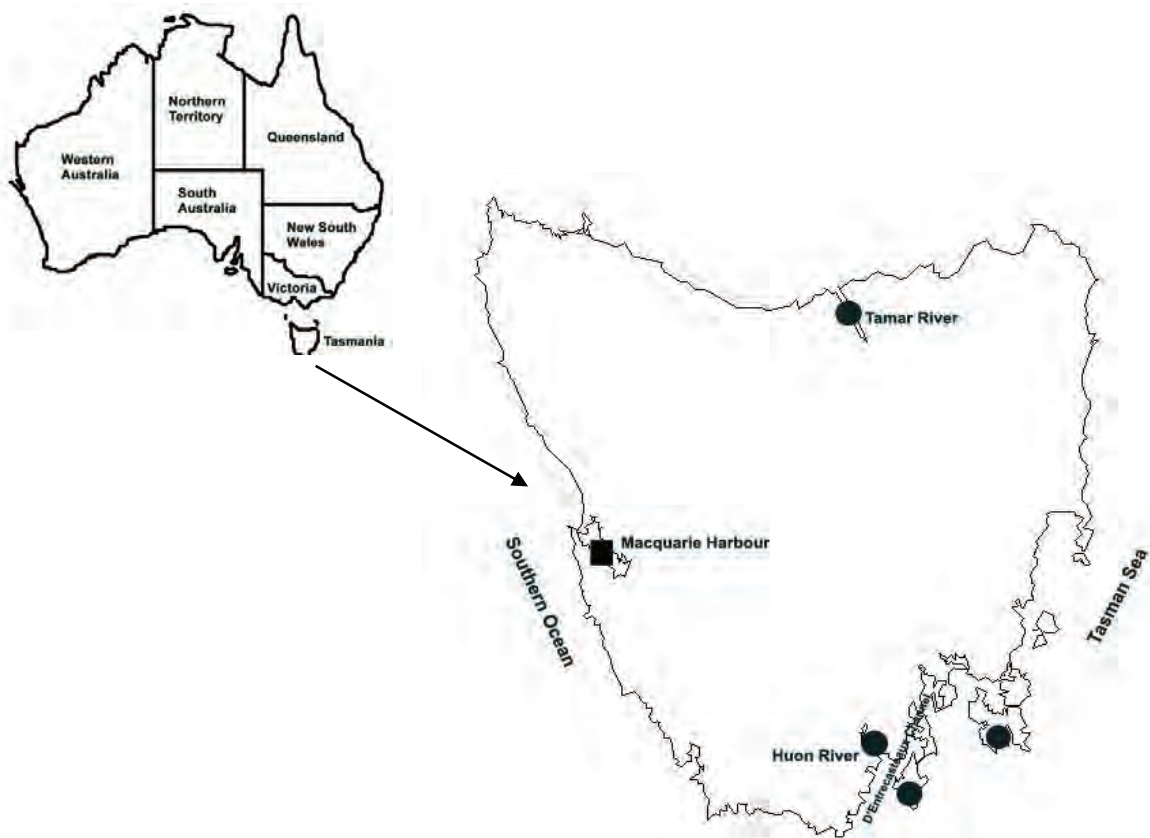


Figure 1.3 Map of Tasmania showing the location of AGD-affected (circles) and unaffected (square) salmon farms

Even though Tasmania is the most severely AGD affected salmon producer, AGD outbreaks have also been reported in cultured Atlantic salmon from Chile, France, Ireland and Spain (reviewed by Munday et al. 2001); in coho salmon, *Oncorhynchus kisutch* from Washington State and California, USA (Kent et al. 1988); in chinook salmon, *Oncorhynchus tshawytscha* from New Zealand (Findlay et al. 1998); in cultured turbot, *Scophthalmus maximus* from Spain (Dyková et al. 1995); and *Neoparamoeba* spp. were also isolated from the gills of sea bass, *Dicentrarchus labrax* within the Mediterranean region (Dyková et al. 2000).

1.2 *Neoparamoeba* spp., the aetiological agent of AGD

Several studies on AGD have acknowledged *Neoparamoeba* spp. to be the primary causative agent of AGD (e.g. Nowak 2001; Adams and Nowak 2004). At present two species of *Neoparamoeba* [*N. pemaquidensis* (see Munday et al. 1990, 2001; Adams and Nowak 2004) and *N. branchiphila* (see Dyková et al. 2005)] have been identified as being associated with AGD. *Neoparamoeba* spp. are normally free-living marine protozoans which becomes parasitic under certain conditions (Dyková et al. 1998, 2000, 2005). These amoebae have been isolated from marine and estuarine sediments at various locations around Tasmania, regardless of the presence of salmon farms (Crosbie et al. 2005). While the presence of AGD in farmed fish is common in Tasmania, wild fishes sampled from AGD affected salmon farm locations, were not infected with AGD (Douglas-Helders et al. 2002). Thus far, laboratory infection has only been established by cohabitation of AGD-affected Atlantic salmon with naïve Atlantic salmon (reviewed by Munday et al. 2001) or by

inoculation of naïve Atlantic salmon with fresh amoeba isolates obtained from AGD affected Atlantic salmon (Zilberg et al. 2001; Morrison et al. 2004) because the disease cannot be reproduced using cultured organisms (Kent et al. 1988; Howard et al. 1993; Morrison et al. 2005).

1.3 AGD pathology & diagnosis

AGD-associated mortality has been presumed to be associated with respiratory disturbance (Kent et al. 1988). However, recent results suggest that mortalities in cultured fish are not caused by respiratory failure (Powell et al. 2000) but may be due to cardiovascular dysfunction (Fisk et al. 2002; Powell et al. 2002; Leef et al. 2005a, 2005b). Currently, presumptive AGD diagnosis is confined to the gills where focal or multifocal white mucoid patches and profuse mucus production may be seen as indicative of AGD severity (Figure 1.4; Clark and Nowak 1999). Salmon farmers frequently monitor AGD progression in entire cage populations by scoring the gross gill lesions as light/medium/heavy infection to determine when to treat the disease. Although, this non-destructive method of diagnosis has proven successful in the control of large-scale mortalities, it is unreliable, particularly for lighter cases of infection (Clark and Nowak 1999).

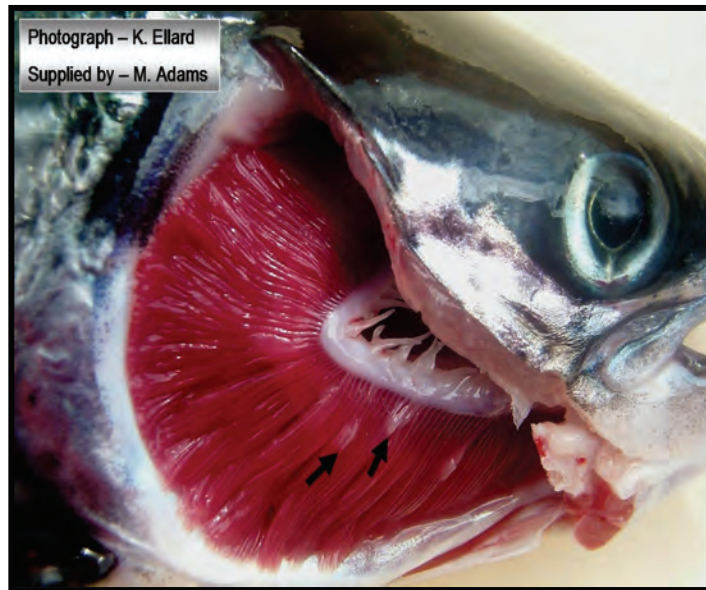


Figure 1.4 Atlantic salmon gills with gross AGD lesions (arrows)

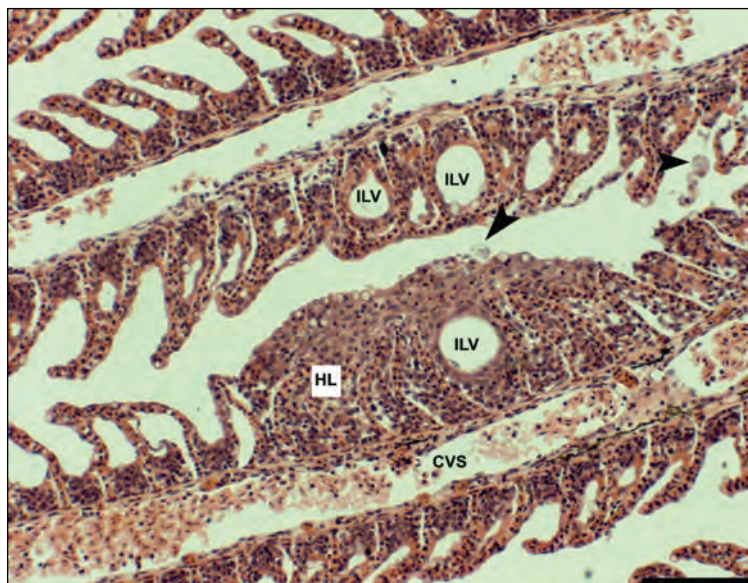


Figure 1.5 Severely infected Atlantic salmon gill showing *Neoparamoeba* spp. (black arrows) on the surface of hyperplastic interlamellar units (HL), interlamellar vesicles (ILV) and infiltration of the central venous sinus with leucocytes (CVS). Scale bar = 100 µm.

For AGD research and diagnostic purposes, histopathology is the only definitive method indicating a diseased state and its causative agent. Therefore, histopathology is considered the 'gold standard' for confirmation of AGD. However, histopathological assessment requires lethal sampling of fish. Microscopic examination of histological sections of AGD-affected fish gills shows macroscopically visible mucoid patches as areas of epithelial cell hyperplasia (Figure 1.5; Adams et al. 2004). Amoebic trophozoites are commonly found closely associated with AGD-induced hyperplastic lesions (Kent et al. 1988; Munday et al. 2001). The degree of histopathology associated with hyperplastic AGD lesions ranges from relatively minor epithelial cell hyperplasia affecting a few lamellae to lesions consisting of multiple fused lamellae and even fused filaments (Zilberg and Munday 2000; Adams and Nowak 2004). Hyperplastic lesions have a spongiotic appearance and consist primarily of hyperplastic undifferentiated epithelial cells with minor oedema (Kent et al. 1988; Roubal et al. 1989; Munday et al. 1990; Dyková et al. 1995). Extensive epithelial cell hyperplasia often causes the formation of interlamellar spaces that frequently contain amoebae associated with leucocytes (Roubal et al. 1989; Munday et al. 1990). As hyperplastic lesions develop, their morphology changes to that of a mature lesion, often developing a layer of stratified epithelial cells that line the lesion surface (Adams and Nowak 2003).

The other non-destructive methods for detecting the presence of amoebae in gill mucus are: (1) immuno-fluorescent antibody test (IFAT), which uses a polyclonal antibody against a Tasmanian isolate, *N. pemaquidensis* strain PA027 (Howard and Carson 1993), (2) Differential Quick Dip[®] staining of gill smears enables easier

distinction between the different cells (Zilberg et al. 1999), (3) detection of amoeboid like cells in wet preparation from fish gills under a light microscope (Clark et al. 2003). Furthermore, a species specific PCR has been developed that provides a highly specific detection and identification assay for *N. pemaquidensis* (Wong et al. 2004) and *N. branchiphila* (Dyková et al. 2005). However, these tests are currently used on cultured amoebae and are yet to be optimized for routine diagnostic purposes.

1.4 AGD treatment & control

Freshwater bathing is the only available effective control for AGD infection in sea-farmed Atlantic salmon. The treatment involves immersing the AGD affected fish for 2-3 h in fresh water to remove/kill the *Neoparamoeba* spp. attached to the gills (Parsons et al. 2001; Clark et al. 2003). Although large scale mortalities are generally avoided by freshwater treatment, this method is both expensive ($\approx 10\%$ of production costs) and production limiting due to the requirement of an unlimited freshwater resource to be located near the lease site. Furthermore, complete removal of the parasite is not achieved and under favourable conditions, AGD can reoccur within 10 days (Clark et al. 2003). Meanwhile, other alternative treatments for AGD has been tested in the laboratory and this includes use of feed supplements (glucans) (Zilberg et al. 2000; Bridle et al. 2005), chemicals such as levamisole (Findlay et al. 2000), oxidative disinfectants such as chlorine dioxide, chloramine-T, hydrogen peroxide (Harris et al. 2004; Powell and Clark 2004) and mucolytic drug such as L-cysteine ethyl ester (Roberts and Powell 2005). However, in commercial situation the outcomes of these treatments i.e. use of feed supplements, chemicals or drugs

have been inconclusive and require further investigation (Clark and Nowak 1999; Zilberg et al. 2000; Harris et al. 2004).

1.5 Factors influencing AGD

The exact environmental conditions and health status of the fish that allow *Neoparamoeba* spp. to proliferate on fish gills are still unknown. There are several factors influencing the occurrence of AGD in Tasmania. Several research studies have been conducted on temperature and salinity and have identified them to be the major risk factor for occurrence of AGD in Tasmania (Munday 1990; Clark and Nowak 1999; Douglas-Helders et al. 2001). However, the effect of other intrinsic and/or extrinsic factors on AGD outbreaks such as salmon stocking density, fish immune status, harm caused by algae, jellyfish or bacteria remains undetermined (Roubal et al. 1989; Lom and Dyková 1992; Nowak and Munday 1994; Findlay and Munday 1998; Findlay et al. 2000; Zilberg and Munday 2000; Nowak 2001; Bowman and Nowak 2004; Douglas-Helders et al. 2005b).

1.6 Interactions between bacteria & amoebae

Previous studies have reported the associations and resultant impacts between pathogenic amoebae and bacteria. For example, the ability of *Entamoeba histolytica* strains to destroy monolayers of cultured cells was enhanced after incubation with various types of Gram negative bacteria (Bracha and Mirelman 1984). Bottone et al. (1992) reported that the growth of *Acanthamoeba castellanii* and *A. polyphaga* was increased in the presence of bacteria and also suggested that the bacterial cocontaminants of contact lens care systems might have a secondary role in the

pathogenesis of amoebic keratitis (a vision threatening disease in humans that occurs due to colonisation of *Acanthamoeba* spp., mainly in contact lens users). In addition, some case series and case reports on *Acanthamoeba* keratitis, have described mixed-infection with *Acanthamoeba* and bacteria (Bacon et al. 1993; D'Aversa et al. 1995). Some amoebae-resistant-bacteria (ARB) have evolved to become resistant to the destruction (e.g. digestion) by free-living amoebae and are able to survive, grow and exit free living amoeba after internalization; these bacteria could be obligate intracellular bacteria or facultative intracellular bacteria (reviewed by Greub and Raoult 2004). *Acanthamoeba* spp. are known to provide an intracellular niche for spore formation and survival for several obligate bacterial endosymbionts including *Chlamydia* spp. (Amann et al. 1997; Essig et al. 1997; Fritsche et al. 1999, 2000); *Legionella pneumophila* (Berk et al. 1998); *Coxiella burnetii* (La Scola and Raoult 1996); *Burkholderia pseudomallei* (Inglis et al. 2000); *Simkania negevensis* (Kahane et al. 2001); *Mycobacterium avium* (Steinert et al. 1998). An intra-amoebal growth environment enhances the virulence of *Legionella pneumophila* (Cirillo et al. 1999) and coinoculation of *L. pneumophila* with *Hartmannella vermiformis* amoebae increases the pathogenesis of bacteria in causing a lung infection in mice (Brieland et al. 1996, 1997).

Despite these extensive research highlighting the relationships between bacteria and pathogenic amoebae of humans, a paucity of information exists concerning the interactions between bacteria and *Neoparamoeba* spp.. Roubal et al. (1989) observed small foci of bacteria in the host-parasite interface on some *Neoparamoebae* infected gill filaments and therefore suggested further studies to

verify the role of gill colonising bacteria in AGD. Douglas-Helders et al. (2003) reported a co-existing *Flavobacterium* infection on AGD-affected salmon gills during a laboratory infection trial conducted to examine the infectivity of *Neoparamoeba* spp. overtime. Recently, Bowman and Nowak (2004) examined the gill bacterial communities of AGD-affected and unaffected Atlantic salmon using culture-independent 16S ribosomal RNA analyses and suggested that gill bacteria might play a direct role by predisposing the fish to AGD, to exacerbate AGD, or if bacteria are present in increased numbers in water, might be coincident with AGD outbreaks. Lom and Dyková (1992) reported that amphizoic amoebae typically only colonise the gills of immunocompromised fishes, or individuals showing an existing bacterial infection, which might provide a ready food source for amoebae growth. However, the association between salmonid gill bacteria and AGD is yet to be elucidated.

1.7 Thesis aims & outline

The broad aim of this thesis was to investigate the role of salmonid gill bacteria in AGD. This work forms one part of several studies within the Cooperative Research Centre for Aquaculture (Aquafin CRC Ltd., Australia) that are undertaken concurrently on AGD in salmonids. The findings from this work should form the basis for future work assessing the combined effect of bacteria and amoeba on fish and in investigating the role of other microorganisms that are recognized as potential factors influencing AGD.

Firstly, as a number of procedures were developed and used continuously during this study they are described separately in chapter 2. Before I began the actual

study it was essential to get some indication on which group of bacteria reside on salmon gills. Hence, gill bacteria were isolated from AGD-affected and unaffected fish gills (chapter 3). The results from chapter 3 showed that the bacteria from the genera *Winogradskyella* and *Staphylococcus* were predominantly present only on AGD-affected fish gills; as a result, it was important to verify the role of these bacteria in AGD. However, prior to investigating the role of a specific bacterium in the development and severity of AGD it was essential to remove potentially confounding bacteria of normal gill microflora. Therefore, a protocol was adapted to disinfect salmonid gills (chapter 4). Subsequently, this protocol was incorporated in all our *in vivo* trials. Chapter 5, reports an experiment that was conducted to verify the influence of bacteria (*Staphylococcus* sp. and *Winogradskyella* sp.) in the incidence and severity of AGD. One of the important outcomes of this experiment (chapter 5) was that the presence of *Winogradskyella* sp. on salmonid gills during the process of *Neoparamoeba* spp. infection could result in severe AGD. Therefore chapter 6, details an experiment that was carried out to further confirm the role of *Winogradskyella* sp. in AGD. While it was evident that the occurrence of certain bacteria on gills could exacerbate AGD condition, it was also important to examine whether these bacteria (*Winogradskyella* spp.) frequently colonised salmonid gills. Therefore, a field based study was conducted for a period of 102 days and fish were sampled on five different time points to monitor the changes occurring in the culturable gill bacterial population in a farm environment (chapter 7). Finally, considering the results and conclusions drawn from the above mentioned experiments and field trials, the significance of bacteria in AGD is discussed in chapter 8.

Chapter 2

General materials & methods

2

General materials & methods

2.1 Isolation of *Neoparamoeba* spp.

AGD can only be initiated by cohabitation of AGD-affected Atlantic salmon with naïve Atlantic salmon or by inoculation of naïve Atlantic salmon with infective *Neoparamoeba* spp. freshly isolated from fish with AGD (Kent et al. 1988; Howard et al. 1993; Morrison et al. 2005). Therefore, for all *Neoparamoebae* challenges, amoebae cells were harvested immediately prior to challenge from the AGD-affected Atlantic salmon held in the School of Aquaculture, University of Tasmania, by a method described by Morrison et al. (2004) modified by addition of antibiotic and antimycotic solution to eliminate bacteria/fungi from the amoebae inoculum (Butler and Nowak 2004). Infected gills were removed from AGD affected Atlantic salmon after euthanasia (anaesthetic overdose at 20 ml l⁻¹ AQUI-S®). Gills were transported to the laboratory in sterile seawater (SS) containing antibiotic and antimycotic solution (5% v/v 5000 IU ml⁻¹ penicillin and 5 mg ml⁻¹ streptomycin solution (Sigma), 1% v/v 10 mg ml⁻¹ gentamycin (Sigma) and 0.25 mg ml⁻¹ amphotericin B (Invitrogen). The gill arches were separated and placed in sterile distilled water and centrifuged at 400 g for 5 min to quickly detach the amoebae from the gill tissue. The supernatant was discarded and the pellets were resuspended in SS and diluted approximately 50 fold, lightly agitated and decanted into several Petri dishes (Figure 2.1). Amoebae were left to adhere to the bottom of the Petri dish

for 1 h, then the liquid and gill debris transferred into new Petri dishes to allow adherence of any remaining amoebae for another hour. The fluid and gill tissue were removed and discarded and the Petri dishes were washed several times with SS to remove mucus and epithelial cells, while the amoebae remained attached to the bottom of the Petri dish (Figure 2.2a). The amoebae were detached by adding 750 μ l trypsin-EDTA solution (0.025% trypsin per 1 mM EDTA; Invitrogen) and by gently tapping the Petri dishes for a minute (Figure 2.2b). The suspension was then pooled and diluted with SS and centrifuged at 400 g for 10 min. The pellets were resuspended in SS and the amoebae were assessed for viability using a trypan blue exclusion assay (Phillips 1973) then counted using a haemocytometer to give the number of viable cells in solution. Lack of culturable bacteria in the amoebae inoculum was confirmed by plating the inoculum on Shieh's and marine agar medium (appendix 1) and incubating at 22°C for 48 h.

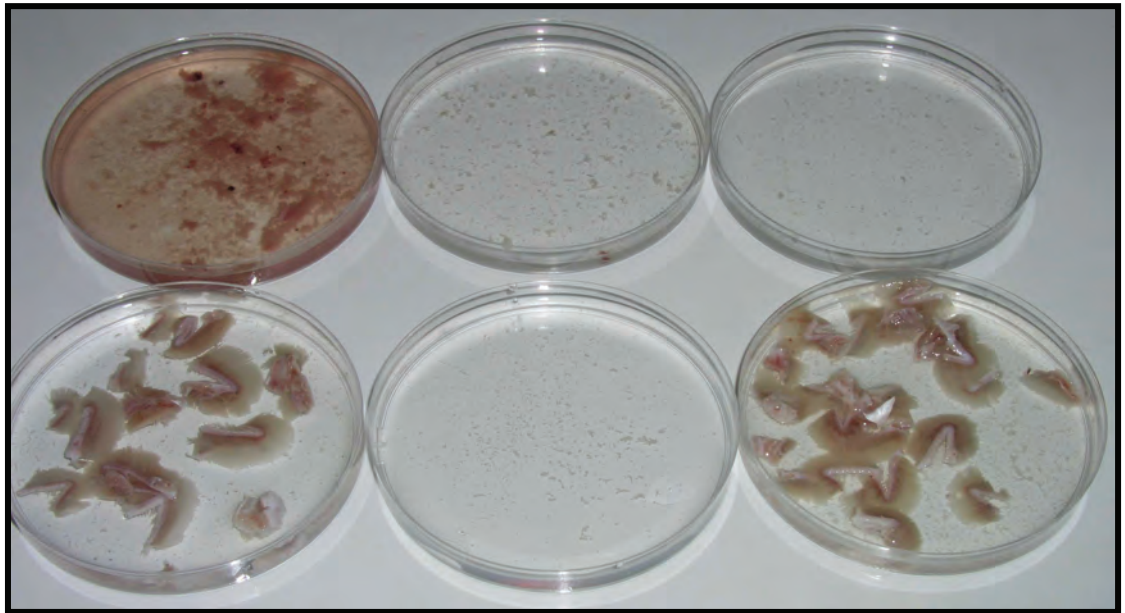


Figure 2.1 Incubation of gill arches in Petri dishes. Photograph by P. Crosbie

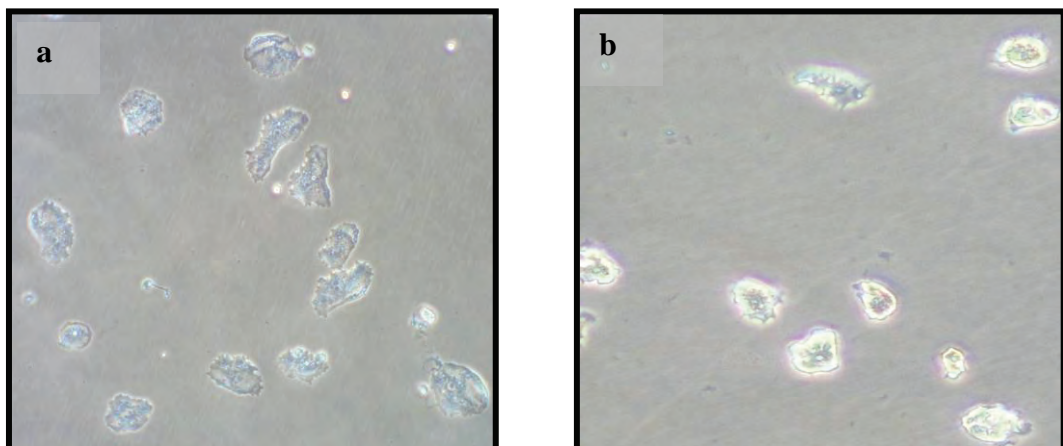


Figure 2.2 Amoebae attached to the bottom of Petri dish 30-60 min after incubation (a) and amoebae detached from the bottom of Petri dish 2-3 min after addition of trypsin/EDTA (b). Photographs by P. Crosbie

2.2 AGD diagnosis and assessment of severity

Fish were sampled with an anesthetic overdose at 20 ml l⁻¹ Aqui-S[®]. The entire gills were removed and placed in seawater Davidson's fixative (Shaw and Battle 1957) and post-fixed in 70% ethanol. The second right gill arch was removed and processed for routine wax histology and stained with haematoxylin and eosin. All sections were viewed by a light microscope (Olympus) at 100 × (for diagnosing AGD lesion percentage) and 400 × (for determining AGD lesion size) magnification. Quantitative analysis of disease severity was conducted by estimating the number and size of AGD lesion on each filament (Adams and Nowak 2001). A typical AGD lesion is characterized by a single or multifocal epithelial hyperplasia of the gill lamellae often containing round to ovate interlamellar vesicles and *Neoparamoeba* spp. with a parasome in association with the lesions. Only well oriented filaments were considered for AGD assessment purpose. A well oriented filament is defined as having >3/4 of the filament with a central venous sinus showing and lamellae evenly sized along both sides of the filament (Speare et al. 1997). The percentage of lesioned filaments was determined by estimating the ratio of filaments with typical AGD lesions to filaments with no AGD lesions. Lesion size was analysed by counting the number of hyperplastic interlamellar units within each lesion. For example, in figure 2.3 there are five filaments of which three (F1, F2 and F3) have AGD lesions; therefore AGD lesion percent would be 60% for this figure; the lesion size in filament F1 is ≈ 5 interlamellar units, F2 is ≈ 26 and F3 is ≈ 23 interlamellar units. Images of the affected tissue were taken using a Leica DC300f digital camera (Wetzlar).

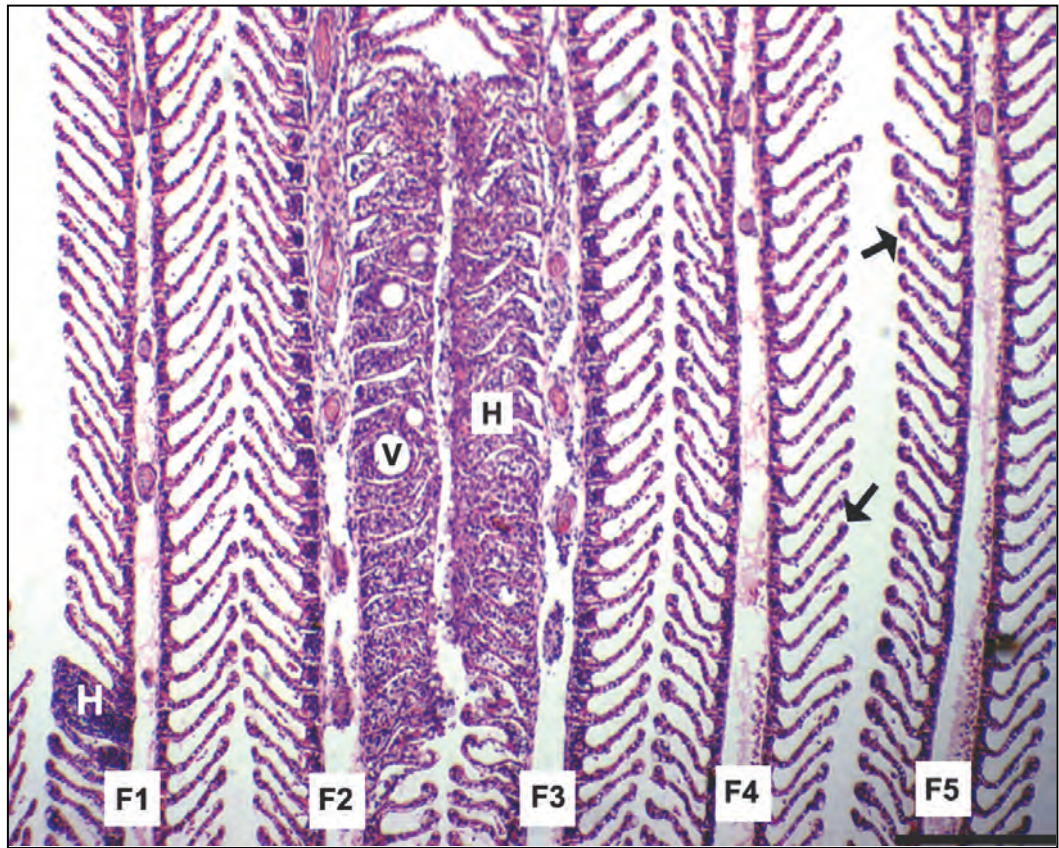


Figure 2.1 A section of Atlantic salmon gills showing AGD-affected filaments (F1, F2 and F3) with hyperplastic lamellae (H) and vesicles (V) and unaffected filaments (F4 and F5) with healthy lamellae (arrows). Scale bar = 250 μ m

2.3 Bacteria isolation and morphological characterisation

Fish were lightly sedated with Aqui-S[®] and gill mucus samples were taken by rotating a swab three times clockwise either from the infected areas showing gross AGD lesions or for fish with no AGD lesions, the mucus samples were collected from second gill arch on the right, in order to be consistent in the sampling procedures. The samples were inoculated onto marine agar and blood agar enriched with 7% sheep's blood and 2% NaCl (chapter 3) or on marine agar alone (chapter 7). Appendix 1 details the formulation for bacteriological agar/broth media used during this study. All plates were incubated at 22°C for 24 to 96 h. Bacterial growth was examined on all plates and distinct bacterial colonies from each plate were chosen according to differences in either one of these morphological characteristics: outline, shape, pigmentation, diameter, transparency, or elevation on agar surface (Figure 2.4). This gave an approximate estimation on the types of culturable bacterial colonies that were present on the fish gills in a given period of time.

To obtain pure cultures, selected colonies from marine agar were inoculated onto fresh plates of Shieh's agar (appendix 1). The colonies selected from blood agar were inoculated onto Todd-Hewitt agar plates (available dehydrated from Oxoid Pty. Ltd., Australia). These plates were incubated at 22°C for 24 to 48 h. The pure colonies thus obtained were cryopreserved (see section 2.4) for further characterisation purposes.

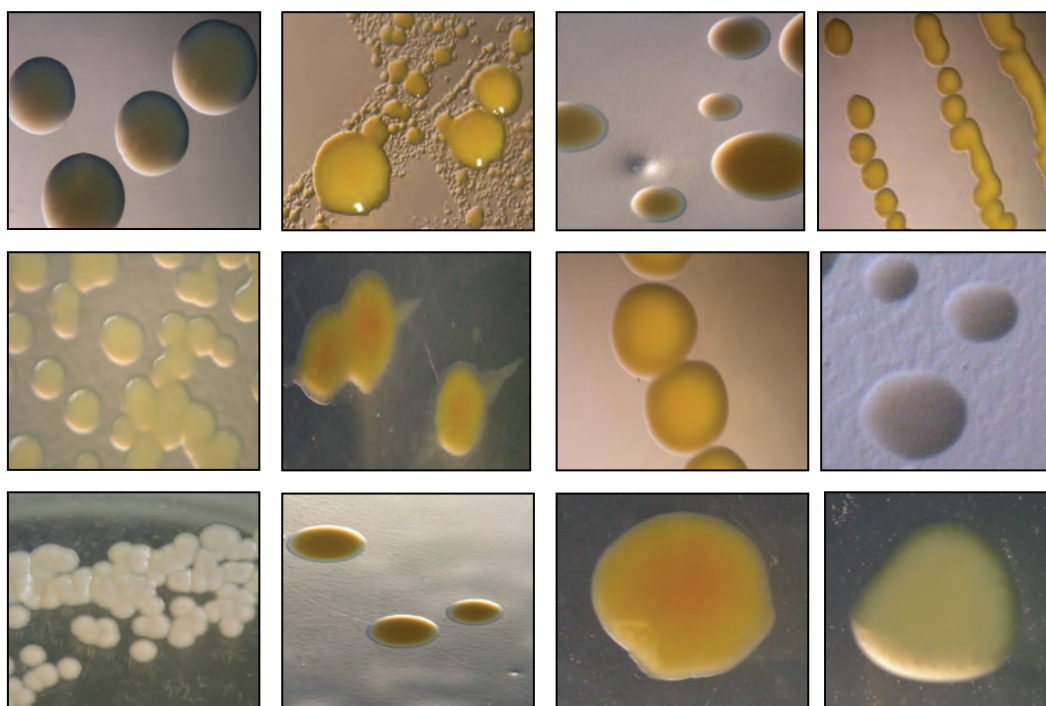
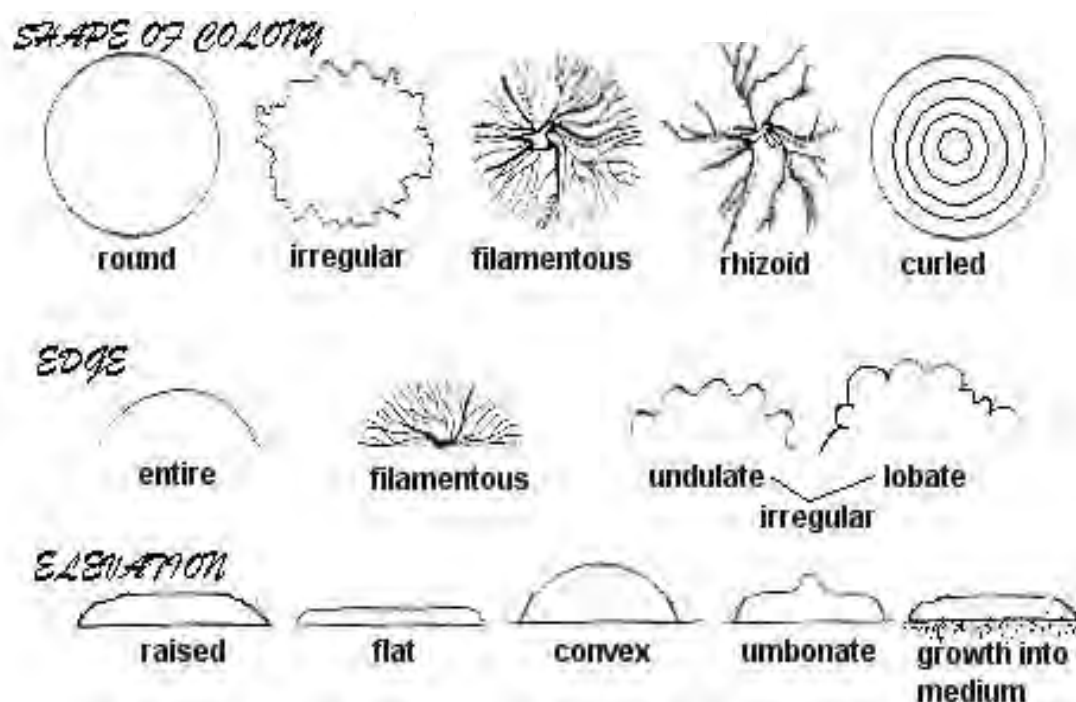


Figure 2.4 Colony Morphology (only sketches not photos copied from website: http://www.rlc.dcccd.edu/mathSci/reynolds/MICRO/lab_manual/ stored under title Colony_morph.jpg) and photographs of some of the bacterial colonies isolated from salmonids gills during the current study.

2.4 Cryopreservation of bacteria

The pure cultures were cryopreserved using the methodology described by Ward and Watt (1971). Briefly, the preservation medium (proteose peptone no.3 (Difco) 1 g; glycerol 8 ml; distilled water 92 ml) was autoclaved at 121°C for 15 min. A dense suspension (100 µl) of the organism, no older than 24-48 h was transferred to a sterile cryotube containing 1 ml of the preservation medium and preserved at -80°C.

2.5 Biochemical characterisation of bacteria

The cryopreserved isolates were grown on their respective culture media (Shieh's or Todd Hewitt agar). The purity of the isolates was ensured before biochemical characterisation. API 50 CH and API CHB/E medium were used to study the carbohydrate fermentation reactions; enzymatic activity of the isolate was studied using API Zym kit (bioMérieux Australia Pty. Ltd.). All tests were conducted according to the protocol supplied with the kits. Values ranging from 0-5 were assigned, corresponding to the colours developed and the values 3, 4 or 5 were considered as positive reactions. API Zym test strips were incubated at 22°C for 4 to 5 h (Figure. 2.5a). API 50 CH test strips were incubated at 22°C for 24 to 48 h (Figure. 2.5b).

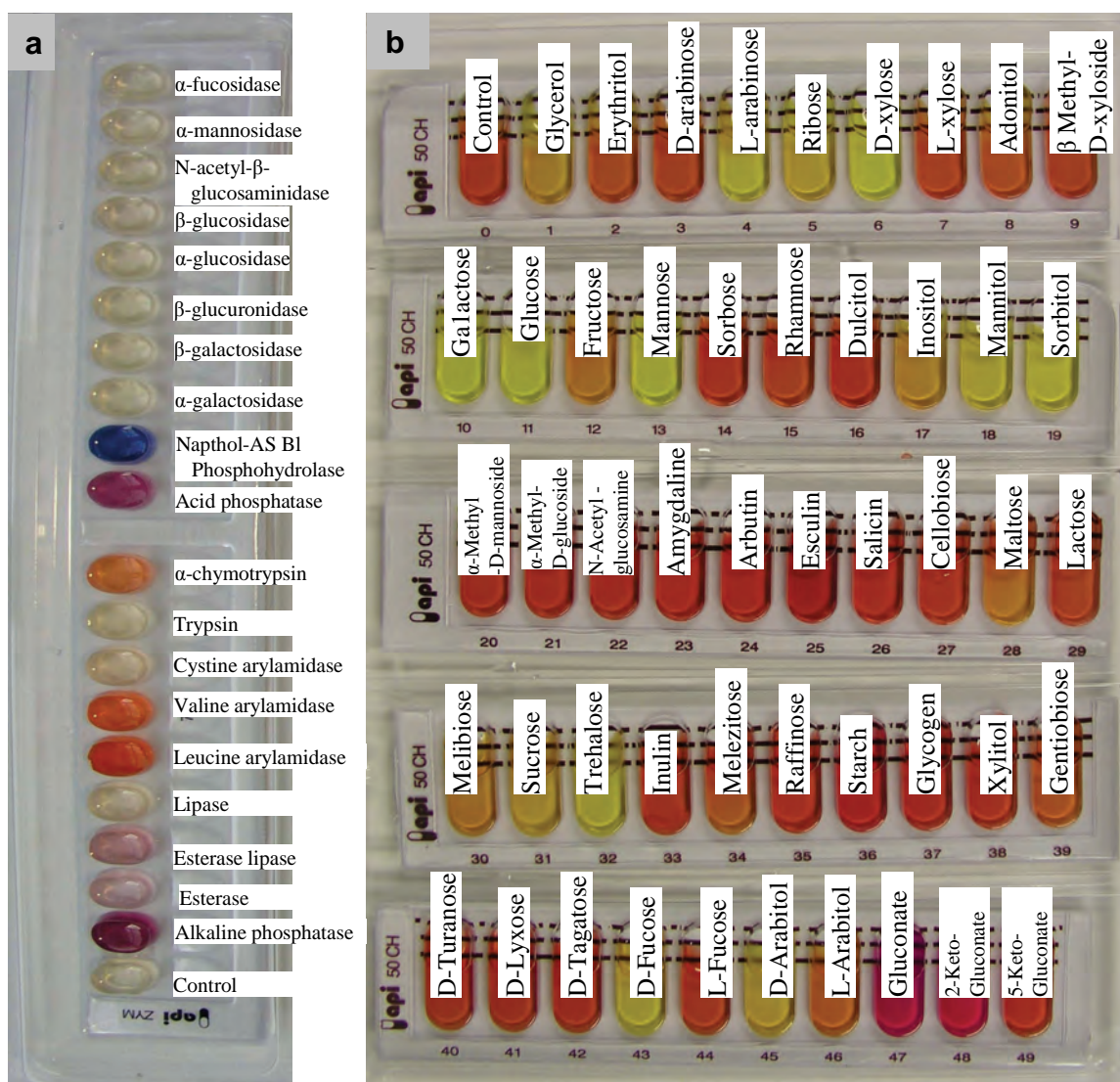


Figure 2.5 API Zym (a) and API 50 CH (b) test results of *Winogradskyella* sp. strain AC1 is shown as an example. Referring to **figure 2.5a**, depending on the intensity of the colour developed compared with control, a value of ≥ 3 was recorded for alkaline phosphatase, esterase lipase (C8), α -chymotrypsin, leucine arylamidase, valine arylamidase, acid phosphatase and napthol-AS B1 phosphohydrolase, indicating that *Winogradskyella* sp. strain AC1 could metabolise these enzymes. Similarly, **figure 2.5b** shows that *Winogradskyella* sp. strain AC1 could form acid with glycerol, L-arabinose, ribose, D-xylose, galactose, glucose, mannose, manitol, sorbitol, melibiose, sucrose, trehalose, D-fucose, D-arabitol, L-arabitol and Gluconate.

2.6 Determination of standard curve

Cryopreserved samples of *Winogradskyella* sp. strain AC1 and *Staphylococcus* sp. strain AC8 were grown on multiple agar plates of Shieh's and Todd Hewitt medium respectively for 48 h at 22°C. The resultant bacterial cells were then aseptically scraped from the agar medium and were serially diluted in autoclaved seawater to obtain a tenfold dilution. The viability of the cells was tested using trypan blue exclusion assay (Phillips 1973) and the numbers of these viable cells in each dilution was determined using standard haemocytometer counting techniques. The corresponding absorbance of the bacterial suspension in each serial dilution test tubes was measured using a spectrophotometer at wavelength 540 nm. Thus, a standard curve was obtained for *Winogradskyella* sp. strain AC1 and *Staphylococcus* sp. strain AC8 (Figure 2.6) by plotting absorbance against cell numbers.

- *Staphylococcus* sp. strain AC8; $y = 0.2381x - 0.0563$. $R^2 = 0.9808$
 ■ *Winogradskyella* sp. strain AC1; $y = 0.1432x - 0.1089$. $R^2 = 0.9822$

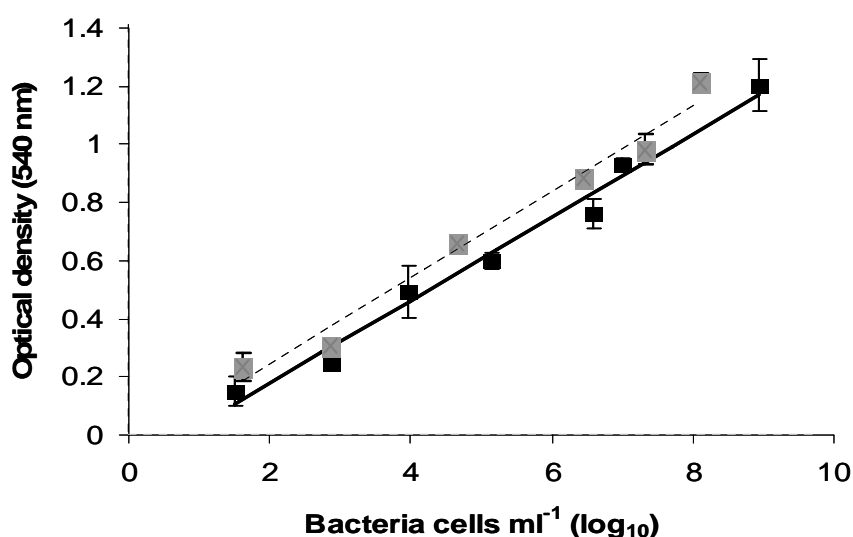


Figure 2.6 Standard curves for *Winogradskyella* sp. strain AC1 and *Staphylococcus* sp. strain AC8. Values are mean \pm SE.

2.7 Cultivation of bacteria for experimental purpose

Cryopreserved samples were grown on their respective culture media (Shieh's or Todd Hewitt broth). Briefly, the bacteria were revived using starter cultures (4 x 500 ml broth) that were placed on an orbital mixer (Ratek Instruments Pty. Ltd., Australia) and rotated at 3-4 rpm for 24 h at $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$ (Figure 2.7a). Broths containing the bacteria were then pooled and transferred to an aerated 3 l carboy and incubated for a further 48 h at $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$ (Figure 2.7b). A stock solution of bacteria was obtained by centrifugation at 10,000 g and by multiple washings with autoclaved seawater. Based on the previously determined standard curve values (see section 2.6) the stock solution was then mixed with suitable volumes of autoclaved seawater to obtain desired challenge concentrations. The viability of the cells was tested using trypan blue exclusion assay (Phillips 1973). Aseptic conditions were adapted throughout the process of bacteria culture.

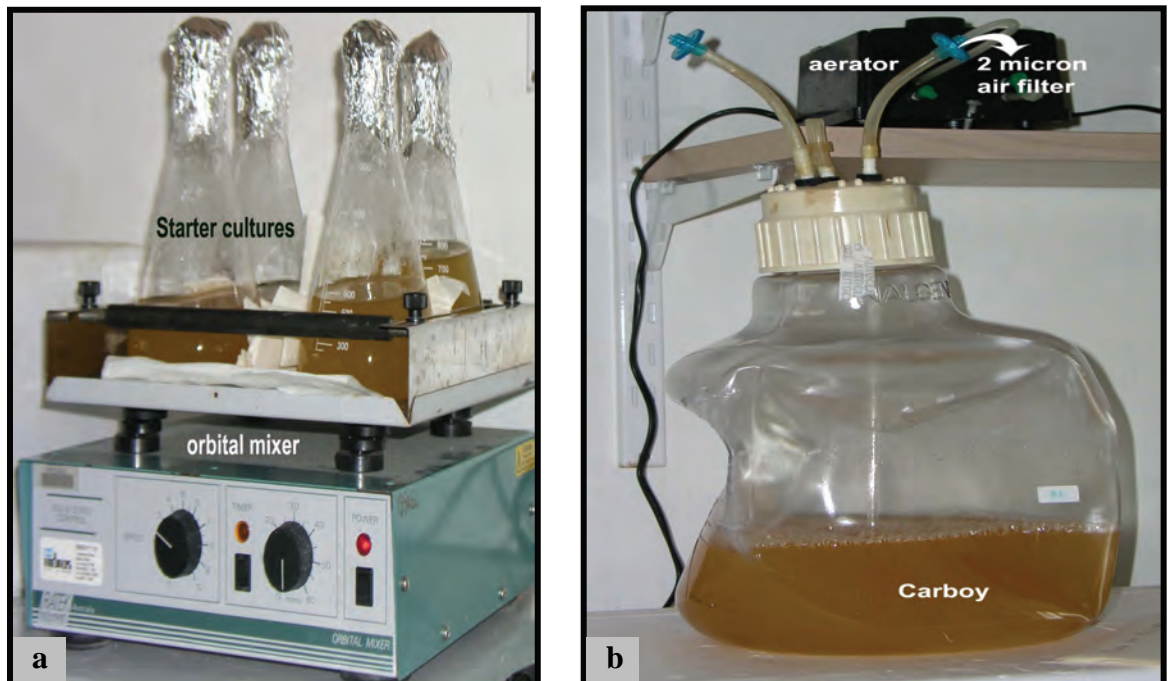


Figure 2.7 Bacteria were revived using starter cultures (a) and after 24 h were transferred into aerated 3 l carboy for large scale production (b).

2.8 Extraction of bacterial DNA

The cryopreserved samples (refer to section 2.4 for details on cryopreservation) were revived on their respective culture medium (Shieh's or Todd Hewitt agar). Bacterial cells were scraped from the agar and suspended in tubes containing 400 µl of saline EDTA, 50 µl of 20% sodium dodecyl sulfate (SDS) and 0.1 mm zirconia/silica beads (Biospec). The tubes were subjected twice to bead beating for 10 seconds at 5000 rpm, and then centrifuged at 20,817 g for 5 min. The aqueous top layer was transferred to a sterile tube and extracted with an equal volume of chloroform-isoamyl alcohol (24:1) and then centrifuged for 10 min at 20,817 g. The DNA thus obtained was purified using the Ultra Clean DNA purification kit (Mo Bio Inc).

2.9 Polymerase chain reaction (PCR)

16S rRNA gene fragments of the DNA extracts were obtained by PCR, using bacteria-specific 16S rRNA gene primers 10F (5'-AGTTTGATCATGGCTCAGATTG-3') (chapter 3) (Weisburg et al. 1991) or 519F (5'-CAGCMGCCGCGGTAATAC-3') (chapter 7) as forward primers (Lane 1985) and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') as reverse primer (chapters 3 and 7) (Lane 1991). PCR was conducted using Hotstar PCR amplification kit (Qiagen) on a Mastercycler[®] thermocycler (Eppendorf AG). The reaction cycle consisted of 95°C for 15 min (initial denaturation); 30 cycles of a minute duration at temperatures 94°C, 52°C, 72°C; a final extension of 72°C for 10 min; and a hold at 4°C. PCR products were assessed by electrophoresis in ethidium bromide-stained 1% agarose gels, visualised with ultraviolet light transillumination with comparison against base pair markers. PCR products of

size, 1.5 kb were purified using the UltraClean PCR Clean-up kit (Mio-Bio Inc.). PCR products were sequenced using the Beckman CEQ2000 automated DNA sequencing system (Beckman-Coulter) using the manufacturer's protocols, except that primers 10F or 519F and 1492R were added in 5 pmol quantities in the sequencing reactions.

2.10 Analysis of sequences

Sequences were manually checked using the programs Chromas v.2.0 (Technelysium Pty.Ltd., Helensvale, Qld, Australia) and BioEdit v.7.0.4.1 (Hall 1999) to confirm individual base positions. Sequence data were aligned to the closest relative using the BLAST database algorithm (Altschul et al. 1997). The sequence dataset was analysed using Clustal X v.1.83 (Thompson et al. 1997) to perform multiple alignments and to construct a tree by the neighbour-joining method. The created trees were viewed in TreeView v.1.6.6 (Page 1996).

2.11 Sequence identification

The criteria established by Bosshard et al. (2003), were followed to assign a 16S rRNA gene sequence to a particular genus and species. Briefly, if the unknown sequence had a homology value of $\geq 99\%$ with the reference sequence of a classified species then the unknown isolate was assigned to this species. Similarly, when the homology value was $< 99\%$ and $> 95\%$, the unknown isolate was assigned to the corresponding genus. When the value was $\leq 95\%$ the unknown isolate was assigned to the corresponding family/order.

2.12 Experimental tanks

The *in vivo* trials (chapters 4, 5 and 6) were conducted in 6 identical recirculating systems each consisting of three 70 l tanks (n=4 fish per tank) and a 70 l reservoir (Figure 2.8). Seawater temperature was maintained at $16 \pm 0.5^{\circ}\text{C}$, pH 8.2 ± 0.5 , dissolved oxygen $7.6 \pm 0.4 \text{ mg l}^{-1}$, salinity 35‰ and total ammonia-nitrogen below 0.2 mg l^{-1} . Sufficient air supply was maintained in the tanks throughout the experiment.

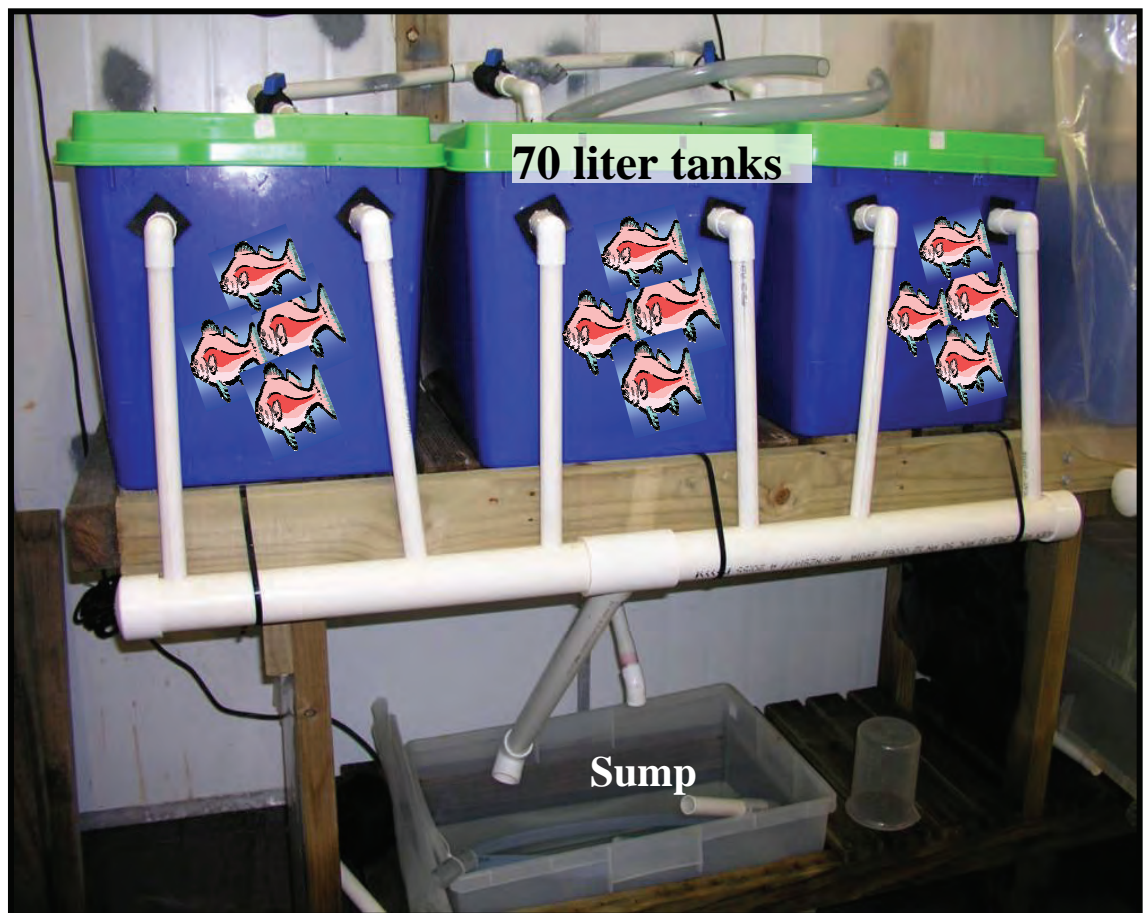


Figure 2.8 A single recirculating system showing three fish tanks and a sump.

2.13 Potassium permanganate treatment

Fish were transferred from recirculation tanks (section 2.12) using dip-nets to individual static tanks for a short term bath (20 min) in seawater (208 l) containing potassium permanganate (5 mg l^{-1}) to remove/reduce the natural microflora on the gills. After the bath, fish were transferred back to their respective recirculation systems and were maintained for 2 days to return to normal conditions. Sufficient air supply was maintained in the tanks throughout the treatment.

2.14 Statistical analysis

A one-way ANOVA using SPSS[®] version 11.5 was performed to test for significant difference between the groups (chapters 4, 5 and 6). Homogeneity of variance was assessed using Levene's test of equality of error variances. If ANOVA's results were significant, then differences between groups were assessed using Tukey's HSD post-hoc test. Differences were considered significant at the $P \leq 0.05$ level. Graphs were drawn using Sigma Plot 2000, SPSS[®] Inc.

Chapter 3

A culture-dependent 16S rRNA gene-based approach
to identify gill bacteria associated with amoebic gill
disease in Atlantic salmon

A culture-dependent 16S rRNA gene-based approach to identify gill bacteria associated with amoebic gill disease in Atlantic salmon

3.1 Abstract

The culturable gill bacterial populations associated with amoebic gill disease (AGD) in Atlantic salmon (*Salmo salar*) were identified using biochemical tests, cluster analysis and 16S rRNA gene-based approaches. The gills of fish with clinical signs of AGD were dominated by isolates that had biochemical profiles similar to the representative strains identified as *Winogradskyella* spp. and *Staphylococcus* spp.. Such strains could not be cultured from the AGD-negative samples. This study discusses the possibility of association of culturable salmonid gill bacteria in AGD.

3.2 Introduction

Amoebic gill disease (AGD) is the major health problem affecting marine farmed Atlantic salmon, *Salmon salar* L., in Tasmania, Australia. The causative agent of AGD is *Neoparamoeba* spp. (reviewed by Munday et al. 2001; Dyková et al. 2005). Amoebae infect the gills of salmon and cause lesions characterised by single or multifocal epithelial hyperplasia of the gill lamellae, focal fusion of secondary lamellae often containing round to ovate interlamellar vesicles and amoebae (Adams and Nowak 2004). At present, the only effective control measure for AGD is freshwater bathing (Parsons et al. 2001; Clark et al. 2003).

Previous studies on the microflora of fish gills of both marine and freshwater fishes have shown that the gills support quite high populations of a wide range of bacterial genera (Trust 1975; González et al. 1999; Bowman and Nowak 2004). Involvement of gill colonising bacteria in AGD has previously been suggested (Roubal et al. 1989; Douglas-Helders et al. 2003), however very few studies have been conducted to verify any role of bacteria in AGD. Bowman and Nowak (2004) conducted a culture-independent 16S rRNA gene-based approach to examine the entire bacterial community on AGD-affected and unaffected salmon gills and proposed that the bacteria from the genus *Psychroserpens* may be linked to the disease. However, this proposition was merely based on the predominant occurrence of *Psychroserpens* spp. on AGD-affected fish gills. To prove the association of bacteria in AGD, it is necessary to conduct *in vivo* trials; and to conduct infection trials it is essential to isolate bacteria because it is not possible to use unculturable bacteria for experiments. As a result, a culture-dependent study needs to be conducted to isolate gill bacteria from AGD-affected Atlantic salmon, in order to test the role of bacteria in AGD. This study is the first attempt to characterise gill-associated bacteria cultivated from salmon with AGD.

3.3 Materials and methods

3.3.1 Fish

Three groups of Atlantic salmon, designated as A, B and C were used in this experiment. Group A fish (n=24) with clinical AGD were from commercial sea cages located in southern Tasmania. Group B and C fish were reared in same farm environment and transported to the aquaculture facility at University of Tasmania, Launceston, Australia, when the smolts were ready to be transferred to sea water. At the time of sampling, group B fish (n=5) were located in the experimental AGD infection tank of 4000 L capacity filled with 2 µm filtered sea water. These fish in Group B tank act as a reservoir for maintaining constant supply of fresh isolates of virulent *Neoparamoeba* spp. required for various AGD infection trials conducted in this facility. AGD could not be reproduced using cultured *Neoparamoeba* spp. because the cultured amoebae are avirulent (Morrison et al. 2005). Group C (n=18) comprised fish naïve to AGD housed in similar 4000 L tanks. Seawater temperature was 16-18°C and salinity was 35‰ at both locations (farm and School of Aquaculture). The naïve fish were selected only from laboratory, because it is not possible to obtain seawater reared Atlantic salmon that are naïve to AGD from Tasmanian aquaculture farms with similar environmental conditions to the AGD positive farms. The farms situated on Macquarie Harbour are AGD negative (see chapter 1, section 1.2); however, environmental conditions in Macquarie Harbour are very unusual and Atlantic salmon farmed there are affected by pathogens specific only for that area.

3.3.2 Sample collection

All fish were lightly sedated with Aquí-S[®] and gill mucus samples were taken by rotating a swab three times clockwise either from the infected areas showing gross AGD lesions (group A and B) or for group C fish that did not have any AGD lesions, the mucus samples were collected from second gill arch on the right, to be consistent in the sampling procedures. Immediately after collection, the samples were inoculated onto marine and blood agars (refer to chapter 2, section 2.3 for details about bacteria cultivation). Gill mucus smears were made for indirect fluorescent antibody testing (IFAT) to confirm AGD infection (Howard and Carson 1993).

3.3.3 Bacterial characterisation

Bacterial growth was examined on all plates and distinct colonies from each group (A, B and C) were chosen according to differences in morphological characteristics (refer to chapter 2, section 2.3 for details on bacteria characterisation). Thus, 50 isolates were selected from Group A, 16 isolates from group B and 52 isolates from group C. The selected colonies were sub-cultured to ensure purity before biochemical characterisation (refer to chapter 2, section 2.3 for details on subculturing procedure). API 50 CH kit and API 50 CHB/E medium were used to study the carbohydrate fermentation reactions; enzymatic activity of the isolates was studied using API Zym kit (bioMérieux Australia Pty. Ltd.) All tests were conducted according to the protocol supplied with the kits (chapter 2, section 2.5 shows in detail the API test results of a single isolate, same procedure was applied for all isolates).

3.3.4 Cluster analysis

The Clustan Graphics software version 5.25 (Wishart, 1999) was used to cluster phenotypically similar bacterial isolates from each group (A, B and C). Results of the 68 API tests for each analysed isolate were recorded in a binary system. Proximities between each isolate were calculated using Squared Euclidean Distance. A hierarchical cluster analysis was obtained using Increase in Sum of Squares (Ward's Method). Based on proximity values of the individual cases, upper tail significance tests created cluster partitions that were significant at the 5% level. From each cluster, the cluster-exemplar was selected from every group and cryopreserved (refer to chapter 2, section 2.4 for details on cryopreservation of bacteria) for subsequent 16S rRNA gene sequence analysis. An exemplar is the most typical member of the cluster with highest mean similarity with all the other members of that cluster (Wishart, 1999).

3.3.5 16S rRNA analysis

The cryopreserved samples were revived on their respective culture medium and were used for sequencing the 16S ribosomal RNA (rRNA). Please refer to chapter 2, sections 2.8, 2.9, 2.10 and 2.11 respectively for details on extraction of bacterial DNA, polymerase chain reaction (PCR), analysis of sequences and sequence identification. Sequences obtained in this study have been deposited under GenBank accession numbers DQ356484 to DQ356511.

3.4 Results

Using IFAT, fluorescent bodies consistent in shape and size to cells of *Neoparamoeba* spp. were recorded in the gill mucus smears obtained from fish in groups A and B, thus confirming that these fish were AGD-positive. No *Neoparamoebae* were detected on the gills of group C fish, the negative controls (data not shown).

From each group (A, B and C) the bacterial isolates with most similar biochemical characteristics were combined to form a cluster that is significant at 5% level. As a result, 10 clusters were formed in group A (AC1 to AC10), 4 clusters in group B (BC1 to BC4) and 14 clusters in group C (CC1 to CC14) (Figures 3.1a to 3.1c). One representative strain of each cluster was selected for 16S rRNA gene sequencing (Table 3.1; Figure 3.2). These representative strains were the cluster exemplars, i.e. these strains were the most typical member of the cluster with highest mean similarity with all the other members of that cluster (Figures 3.1a to 3.1c). Therefore, the biochemical profile of the representative strain would be similar to the rest of the members in the cluster (Table 3.2; Appendix 2). For example, according to 16S rRNA analysis the strain selected from cluster AC1 had >95% and <99% similarity with the phylotypes present in the genus *Winogradskyella*. Therefore, we propose that the remaining 9 isolates in this cluster (AC1) would also be closely related to the genus *Winogradskyella* (Table 3.1; Figure 3.1a).

Based on the above statements, it was found that *Winogradskyella* spp. (isolates from clusters AC1, AC2 and BC1) and *Staphylococcus* spp. (isolates from clusters AC6 to AC10 and BC2 to BC4) predominantly colonised the gills of AGD-affected fish (groups A and B) regardless of fish origin i.e. group A fish were from farm and group B fish were from laboratory. These bacteria were not isolated from AGD-negative fish gills in group C. The gills of group C fish mainly contained *Gammaproteobacteria* [*Idiomarina* spp. (CC2, CC3 and CC4); *Shewanella baltica* (CC1); *Pseudomonas jessenii* (CC5); *P. putida* (CC6); *Cobetia marina* (CC8) and *Halomonas* sp. (CC7)]. The other bacteria isolated during this study include those from the genera *Polaribacter* (AC3 and AC4), *Salegentibacter* (AC5), *Cellulophaga* (CC11, CC12, and CC13) and *Arthrobacter* (CC9) (Table 3.1; Figure 3.2).

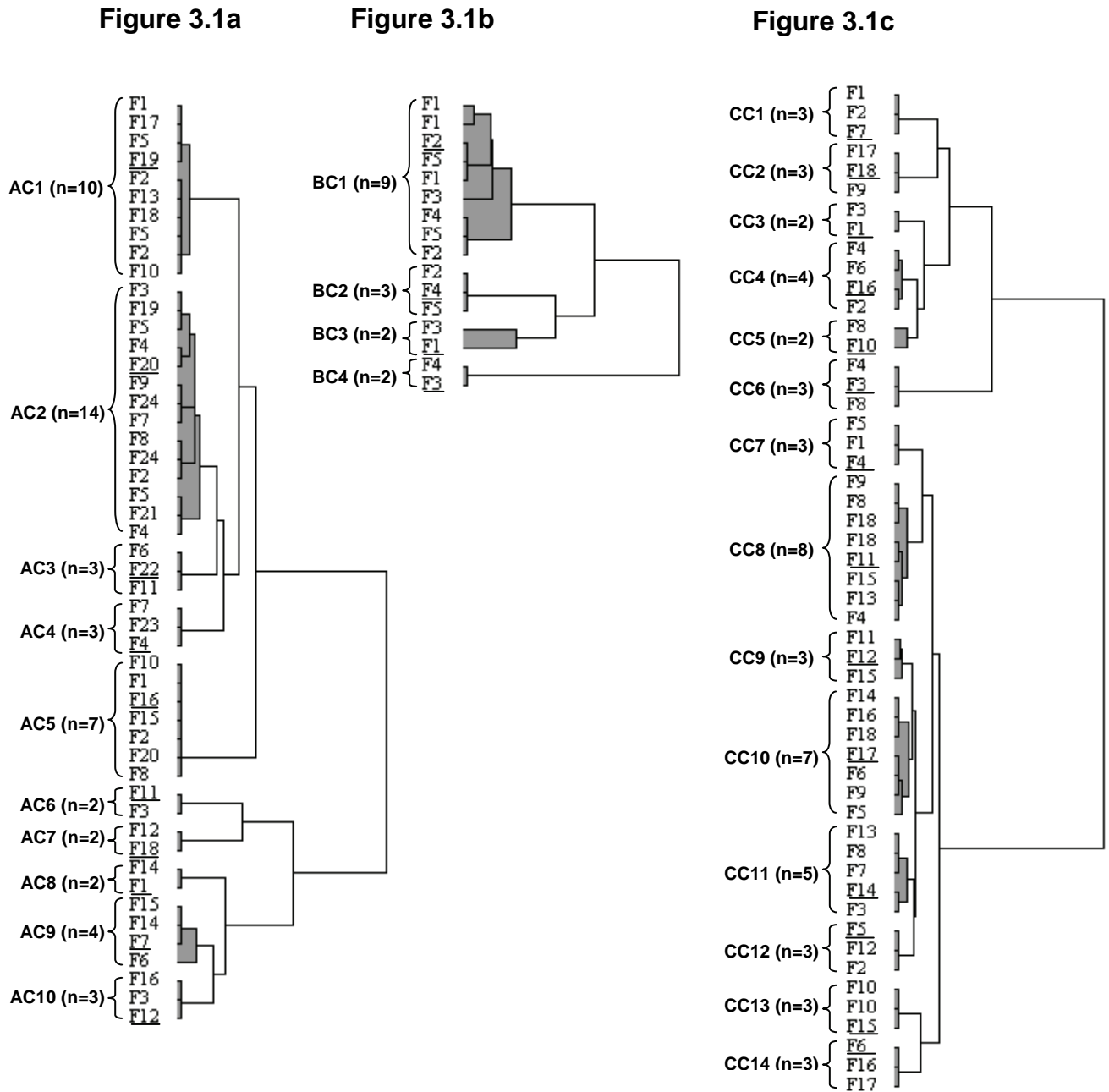


Figure 3.1 Hierarchical comparison of the biochemical characteristics of bacterial strains isolated from AGD-affected and unaffected fish gills. Clusters are denoted by left braces “{” and labeled as (Group A-Cluster 1 (AC1) to Group A-Cluster 10 (AC10); BC1 to BC4 and CC1 to CC14). Bacterial strains isolated from fish gills are designated as F1, F2, F3 and so on. Underlined strains are cluster exemplars, which were selected for 16S rRNA analysis. **Figure 3.1a:** Group A (AGD-affected farm fish); **figure 3.1b:** Group B (AGD-affected fish from laboratory); **figure 3.1c:** Group C (AGD-negative fish from laboratory)

Table 3.1 16S rRNA gene phylotype identity of cluster representative of strains selected from AGD-affected (groups A and B) and unaffected (group C) Atlantic salmon gills

Cluster representative with GenBank accession #	Sequence data with GenBank accession #	% identical 16S rRNA [†]
<u>Group A</u>		
AC1 (DQ356488)	<i>Winogradskyella eximia</i> (AY521225)	96.6%
AC2 (DQ356489)	<i>Winogradskyella eximia</i> (AY521225)	96.9%
AC3 (DQ356492)	<i>Polaribacter irgensii</i> (AY771712)	94.6%
AC4 (DQ356493)	<i>Polaribacter irgensii</i> (AY771712)	95.3%
AC5 (DQ356491)	<i>Salegentibacter salegens</i> (M92279)	99.7%
AC6 (DQ356501)	<i>Staphylococcus pasteurii</i> (AB009944)	99.8%
AC7 (DQ356497)	<i>Staphylococcus warneri</i> (L37603)	99.7%
AC8 (DQ356495)	<i>Staphylococcus kloosii</i> (AB009940)	99.2%
AC9 (DQ356496)	<i>Staphylococcus warneri</i> (L37603)	99.4%
AC10 (DQ356498)	<i>Staphylococcus warneri</i> (L37603)	99.5%
<u>Group B</u>		
BC1 (DQ356490)	<i>Winogradskyella eximia</i> (AY521225)	96.8%
BC2 (DQ356499)	<i>Staphylococcus pasteurii</i> (AB009944)	99.7%
BC3 (DQ356500)	<i>Staphylococcus pasteurii</i> (AB009944)	99.9%
BC4 (DQ356502)	<i>Staphylococcus xylosus</i> (D83374)	99.5%
<u>Group C</u>		
CC1 (DQ356507)	<i>Shewanella baltica</i> (AJ000214)	97.8%
CC2 (DQ356508)	<i>Idiomarina baltica</i> (AJ440214)	95.2%
CC3 (DQ356510)	<i>Idiomarina</i> sp. (AB167017)	96.4%
CC4 (DQ356509)	<i>Idiomarina loihiensis</i> (AE017340)	99.6%
CC5 (DQ356504)	<i>Pseudomonas jessenii</i> (AY206685)	99.4%
CC6 (DQ356503)	<i>Pseudomonas putida</i> (AY958233)	98.9%
CC7 (DQ356506)	<i>Halomonas</i> sp. (U78786)	98.2%
CC8 (DQ356505)	<i>Cobetia marina</i> (AY628694)	99.9%
CC9 (DQ356511)	<i>Arthrobacter rhombi</i> (Y15885)	98.4%
CC10 (DQ356484)	<i>Arenibacter troitsensis</i> (AB080771)	99.0%
CC11 (DQ356486)	<i>Cellulophaga baltica</i> (AJ005972)	96.0%
CC12 (DQ356487)	<i>Cellulophaga baltica</i> (AJ005972)	97.9%
CC13 (DQ356485)	<i>Cellulophaga baltica</i> (AJ005972)	95.5%
CC14 (DQ356494)	<i>Flavobacterium</i> sp. (DQ239767)	95.0%

[†] obtained from GenBank nucleotide database

Figure 3.2 16S rRNA gene-based phylogenetic tree indicating the location of bacterial isolates cultured from the gills of Atlantic salmon (isolates AC1 to AC10 are from AGD-affected farm fish gills in group A; BC1 to BC4 are from AGD-affected fish from laboratory and CC1 to CC14 are from AGD-negative fish from laboratory). Scale bar indicates maximum likelihood evolutionary distance. Numbers in parenthesis are GenBank accession numbers. Figure 3.2 shown in next page.

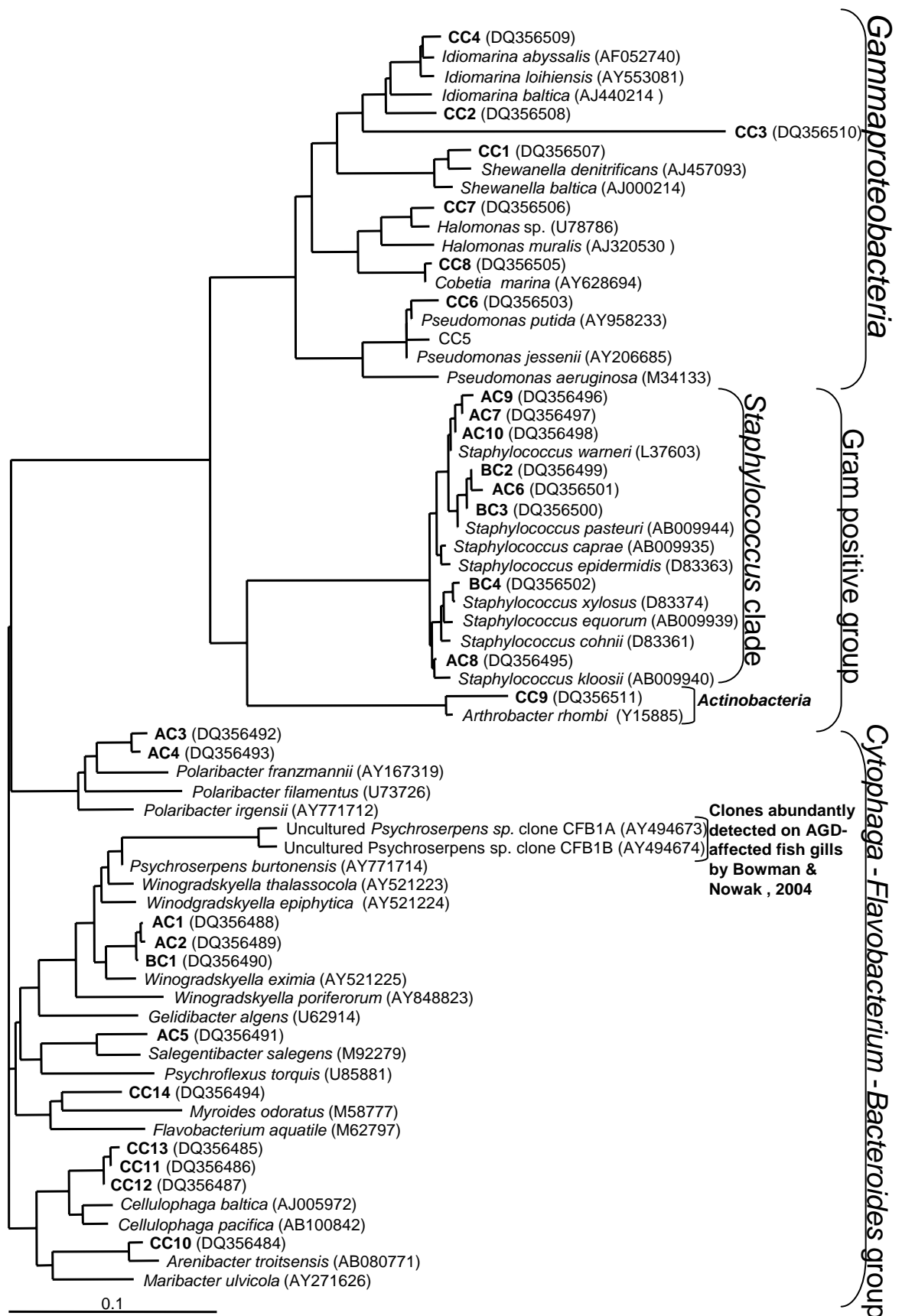


Table 3.2 Biochemical profiles of the cluster exemplars that were selected for 16S rRNA analysis from each cluster in group A (AGD affected fish from farm), group B (AGD affected laboratory fish) and group C (unaffected fish). Cluster-exemplars from group A are denoted as AC1 to AC10; group B as BC1 to BC4 and group C as CC1 to CC14. The numbers represent the percentage of isolates that showed a positive reaction. + denotes a positive reaction by cluster exemplar.

API-test	AC1	AC2	AC3	AC4	AC5	AC6	AC7	AC8	AC9	AC10	BC1	BC2	BC3	BC4	CC1	CC2	CC3	CC4	CC5	CC6	CC7	CC8	CC9	CC10	CC11	CC12	CC13	CC14
Alkaline phosphate					+100	+100						+100	+100	+100	+100	+100	+100	+100	+100	+66	+100	+100	+100	+100		+100	+100	+66
Esterase C4	+100	+64				+100	+100	+100	+100	+100	+88	+100	+100	+100		+100		+100				25	+100		+100		+100	
Esterase Lipase C8	+100		+100	+66	+28	+100	+100	+50	25	+100	33	+100	+100	+100	+100	+100		+100	+100			+63		+100	+100	+100	+100	
Lipase												+100			+100	+100		+100		+100	+100			+100				
Leucine arylamidase					+42	+50	50	+100	+25	33		33	+50	+100	+100	+100	+100		+100			+75						+100
Valine arylamidase			+33	+66		+50		+100	+50	33		+33	+50	+100	+100	+100		+100	+100			+63	33					
Cystine arylamidase																+100							33					
Trypsin										+66					+100				+100		+100				60		+66	+100
α -chymotrypsin														+100	+100	+100			+50			25						
Acid phosphatase	+100		+33	66		+100	+100			33		+100	+100	+100	+100	+100	+100	+100	+100	+33	+100	+100		14		+100		
Naphthol-AS BL Phosphohydrolase	+100					+100	+100	+100	+100		11	+100	+100	+100	+100	+100	+100	+100	+100	+66			+100	43			+33	+100
β -galatosisidase																						+100	+100		20	+66		
β -glucuronidase				66														+50						43	20			
α -glucosidase					+100			50																+43				
N-acetyl- β -glucosaminidase										33		+100			+100			+50	+100					43		33		+100
α -mannosidase																					33				40		33	
α -fucosidase																					33						33	
Glycerol			+100	+100	+100	50		50				+66		+100			+100	+100	+100		+100	+100	+100				+100	+100
Erythritol														+100				+75								33		
D Arabinose			+100												+100		+100					+100		+100	+100	+100	+100	
L Arabinose			+100		+100	50	+50					33	+50		+100					+100	+100	+100	66	+100	+100	+100	+100	
Ribose																					+100							
D Xylose					+100													25		+100	+100	+100			+40		+100	
L Xylose																		25				+100			40		+100	
Adonitol																					+100				40			+100
Galactose			+100	+100																	+100	+100			+100		+100	
Glucose	+100	+100	+100	+100	+100	+100	+100	+100	+100	+100	+100	+100	+100	+100	+100	+100			+100		+100	+100		+100	+100	33	+100	
Fructose		+64		33		+100	+100	+100	+100	+100	+66	+100	+100	+100					+100		+100	+100				+100	+100	
Mannose	+40		+100		+100	+100	+100		+100		44		+100	+100	+100	+100	+100			+100	+100	+100	33	+100	+100	+100		+66

API-test	AC1	AC2	AC3	AC4	AC5	AC6	AC7	AC8	AC9	AC10	BC1	BC2	BC3	BC4	CC1	CC2	CC3	CC4	CC5	CC6	CC7	CC8	CC9	CC10	CC11	CC12	CC13	CC14
Sorbose				+100								+100		+100	+100				+50									
Rhamnose			+100				+100							+100		+100												
Dulcitol																	+100						66					
Inositol							+50							+50								38						
Mannitol		+100		33		+100		+100	+100		+100	+100	+100	+100		+100				+100	+100			14		+100	+100	
Sorbitol						+50		+50	+75				+100	+100	33		+100											
α-Methyl D Mannoside															66								33					
α-Methyl D Glucoside															66													
Arbutin																										33		
Esculin										+66				+100								50	66			33		
Cellobiose	+100				+100																			+100		+100		
Maltose	+100	+100	+33	66	+100						+100			+50	+100	+100	+100					+100		+100	+100	+100	+100	
Lactose			+66											+50		+100	+100	+100			+100	+100			+100		+100	+100
Melibiose														+100		+100	+100							14				
Sucrose		+100	+66		+28	+100	+100	+100	+100	+100	+100	+100	+100	+100	+100	+100	+100				+100	+100		+100	+100	+100	+100	
Trehalose		+64	33	33	28	+100	+100		+100	+100	66	+100	+100	+100	33	33	+50	+75		+100	+100	+100						
Melezitose				33							22			+100	33						+100		33					
Raffinose																					+100		+33		+100		+100	
Strach																						+38						
Glycogen														+100														
Xylitol															33			+100			+100							
Gentiobiose							+50									66		+50			+100		+66					
D-Turanose			33																		+100							
D-Lyxose																					+100							
D-Tagatose																33												
D-Fucose												+100					+50				+100			14	+100	+100		+100
L-Fucose												+100												+14	+100	+100		+100
D-Arabitol						+50											+50											
L-Arabitol																	+50											
Gluconate						+50							+50						+100									
2-KetoGluconate																		+100	+100	+100			33					+100
5-KetoGluconate																		+100										+100
No. of isolates per cluster	10	14	3	3	7	2	2	2	4	3	9	3	2	2	3	3	2	4	2	3	3	8	3	7	5	3	3	3

3.5 Discussion

The present study identified *Polaribacter* spp., *Salegentibacter* sp., *Winogradskyella* spp. and *Staphylococcus* spp. only on the gills of AGD-positive fish; these bacteria were absent on AGD-negative samples. AGD-negative fish had a varied distribution of *Gammaproteobacteria* and *Cellulophaga* spp. These results show that fish with AGD may have a diverse/dissimilar gill bacterial flora compared to fish without AGD.

The current study abundantly isolated *Winogradskyella* spp. and *Staphylococcus* spp. only on AGD-positive fish gills (groups A and B). This might suggest that the onset of AGD on Atlantic salmon gills might favour the growth of certain bacteria. These bacteria might act as secondary pathogens or alternatively, the occurrence of these bacteria in large numbers on the gills might influence *Neoparamoeba* spp. colonisation.

Additionally, several studies on human diseases (e.g. amebiasis, *Acanthamoeba* keratitis) have reported intriguing relationship between bacteria and amoeba. For example, pre-incubation of *E. histolytica* with certain Gram negative bacteria markedly enhanced the virulence of amoeba and their ability to destroy monolayers of tissue-cultured cells (Bracha and Mirelman 1984). Similarly, the growth of *Acanthamoeba castellanii* and *A. polyphaga* could significantly increase in the presence of certain bacteria such as *Xanthomonas maltophilia* (Bottone et al. 1994). Furthermore, some bacteria besides providing nutrition for amoebae might

also survive phagocytosis and are able to multiply within free-living amoebae (Horn et al. 2001). In addition, various strains of *Acanthamoeba* spp. can maintain and transmit internalised pathogenic bacteria such as *Salmonella* sp., *Legionella pneumophila*, *Micrococcus* sp. (Hadaś et al. 2004).

Further research is needed to verify the role of *Winogradskyella* spp. and *Staphylococcus* spp. in AGD. *Winogradskyella* is a recently established marine genus within the family *Flavobacteriaceae*, phylum *Bacteroidetes* (commonly known as *Cytophaga-Flavobacterium-Bacteroides* phylum or CFB group). Currently the genus *Winogradskyella* contains four recognised members [*W. thalassicola*, *W. epiphytica*, *W. eximia* isolated from algal frond surfaces in the Sea of Japan (Nedashkovskaya et al. 2005) and *W. poriferorum* isolated from the surface of a sponge in tropical water (Lau et al. 2005)]. There is no further information available either on the association or the pathogenic potential of *Winogradskyella* spp. with any organism. On the other hand, *Staphylococcus warneri* strains Y-13-L and CECT 236 were reported to be virulent to brown trout, *Salmo trutta* (Gil et al. 2000). Furthermore, a *Staphylococcus* species infection has previously been described in other fish species including silver carp, *Hypophthalmichthys molitrix* (Shah and Tyagy 1986); yellowtail, *Seriola quinqueradiata* and sea bream, *Chrysophrys major* (Kusuda and Sugiyama 1981).

This study also identified a bacterial strain from cluster AC5 closely related to *Salegentibacter salegens* from AGD-affected farm fish. In contrast, the uncultured

Salegentibacter sp. clones were present only on AGD-negative fish gills (Bowman and Nowak 2004). The pathogenicity of *Salegentibacter* species is unknown and only few studies on marine fish gills report the presence of *Salegentibacter* sp.. Similarly, there is no information available on the effect of *Polaribacter* spp. on fish. Bacteria from the genus *Polaribacter* were commonly isolated from Antarctic pack ice (Brinkmeyer et al. 2003).

The culture-independent 16S rRNA gene-based approach conducted to study salmonid gill bacteria on AGD-affected and unaffected fish frequently detected a phylotype (CFB1a) closely related to the members of the genus *Psychroserpens* only on the gills of AGD-affected fish (Bowman and Nowak 2004). However, during the current study, bacteria belonging to the genus *Psychroserpens* were not isolated from the AGD-affected groups A and B. Also, some of the bacteria isolated during this study such as *Winogradskyella* spp.; *Polaribacter* spp.; *Cellulophaga* spp.; *Arenibacter troitsensis* and the *Gammaproteobacteria* [*Idiomarina* spp.; *Shewanella baltica*; *Pseudomonas jessenii*; *P. putida*; *Cobetia marina* and *Halomonas* sp.] were not detected by Bowman and Nowak (2004) during their culture-independent study. Spanggaard et al. (2000) compared the results obtained from both traditional and molecular identification methods and reported that dominant microflora in the fish intestine was generally culturable and was effectively identified by both the methods. One of the possible reasons for not isolating *Psychroserpens* spp. in the current study might be due to the fact that *Psychroserpens* spp. have fastidious growth requirements including a narrow salinity range, requirement of vitamins and amino

acids for growth (Bowman et al. 1997). The other reasons would be differences in fish populations, age, AGD severity and environmental factors such as temperature and salinity.

In summary, this study identified *Winogradskyella* spp. and *Staphylococcus* spp. to be predominant on the gills of AGD-affected fish. These bacteria were absent on AGD-negative samples. Previous studies suggest that certain *Staphylococcus* spp. are pathogenic to fish. There is no information available on the effects of *Winogradskyella* spp. on any organism. Hence, considering the abundance of these bacteria on AGD-affected salmonid gills, the uncertainties about *Winogradskyella* spp. and the pathogenicity of *Staphylococcus* spp., it is worthwhile to investigate the role of these bacteria in AGD.

Chapter 4

Use of potassium permanganate to disinfect Atlantic salmon gills

Published in Bulletin of European Association of Fish Pathologists,
25(6): 291-296, 2005

Please note: The published paper is slightly modified to suite the thesis format and also new AGD literature is incorporated to make this chapter up to date.

Use of potassium permanganate to disinfect Atlantic salmon gills

4.0 Abstract

This study determined the efficiency of potassium permanganate (KMnO₄; 5 mg l⁻¹) in disinfecting Atlantic salmon gills and verified the effect of this disinfection process on subsequent development of amoebic gill disease (AGD). The gills of KMnO₄ treated fish showed a significant reduction in their average bacterial colony forming units (CFU) compared to untreated fish. However, there was no significant difference in AGD severity between KMnO₄ treated and untreated groups.

4.1 Introduction

The free-living amoeba, *Neoparamoeba* spp., parasitizes the gills of marine farmed Atlantic salmon (*Salmo salar*) and causes amoebic gill disease (AGD) (Munday et al. 2001; Dyková et al. 2005). The exact environmental conditions or health status of the fish that allow amoebae to proliferate on fish gills are still unknown (Adams and Nowak, 2004). Previously a culture-dependent (chapter 3) and culture-independent (Bowman and Nowak 2004) study was conducted to examine the salmonid gill bacterial population on AGD-affected and unaffected fish. Bacteria from the genera *Psychroserpens* (Bowman and Nowak 2004), *Winogradskyella* and *Staphylococcus* (chapter 3) were predominantly isolated only on AGD-affected fish gills; therefore, these studies recommended further research to determine the role of

these bacteria in AGD. However, to investigate the role of specific bacteria in the development and severity of AGD, it is desirable to develop a gill disinfection protocol that will reduce potentially confounding bacteria of normal microflora. Potassium permanganate (KMnO_4) was chosen to experimentally disinfect salmon gills because it has been used as a general disinfectant for many years in various aquaculture settings. This compound is an effective bactericide, fungicide and algaecide (Duncan 1974; Jee and Plumb, 1981; Tucker, 1984; Soriano et al. 2000). The aims of this experiment are to evaluate the efficacy of KMnO_4 as a disinfectant and investigate any effect of the KMnO_4 treatment on experimental induction of AGD in Atlantic salmon after exposure to *Neoparamoeba* spp.

4.2 Material and methods

4.2.1 Experimental setup and treatment groups

Atlantic salmon (*Salmo salar*) ($n = 72$), were acclimated to seawater (35‰) over 7 days in 6 recirculating systems (refer to chapter 2, section 2.12 for details about recirculating systems setup and seawater parameters). Following acclimation, fish were divided randomly into three groups of 24 and distributed to duplicate systems per group ($n = 12$ fish per system i.e. 4 fish per tank). Fish in group 1, were exposed to *Neoparamoeba* spp. only; group 2, exposed to KMnO_4 prior to amoebae infection; the 3rd group was exposed to neither KMnO_4 nor amoebae (negative control).

4.2.2 KMnO₄ treatment

Fish from group 2 underwent a short-term bath (20 min) in seawater containing KMnO₄ (refer to chapter 2, section 2.13 for details about KMnO₄ bathing procedure). Groups 1 and 3 were handled in the same way but were bathed in seawater for the same time period. After the recovery period (2 days), a sub-sample of 6 fish from each group were euthanased by Aquí-S[®] (20 ml l⁻¹) and the entire surface of each gill was gently swabbed to collect mucus. Bacteria from the gills were enumerated using the Miles and Misra (1938) inoculation technique; briefly, tenfold dilutions (up to 10⁻⁴ dilution) were prepared in sterile seawater and inoculated onto different segments of marine agar (see appendix 1 for media formulation). Each plate was replicated once. The plates were incubated at 22°C for 24 to 48 h. Total gill bacteria for each sample were determined by counting the numbers of colony forming units (CFU) on each segment of the plate. Results are expressed as the mean number of bacteria per fish. To assess gill damage possibly caused by KMnO₄ exposure, the entire gills were fixed in Davidson's seawater fixative (Shaw and Battle 1957) and then transferred to 70% ethanol. The second right gill arch was wax embedded and 5 µm sections were cut and stained with haematoxylin and eosin. All sections were viewed under light microscope at magnifications ranging from X100 to X400 (Olympus, Hamburg, Germany). To differentiate between the gill damage possibly caused by KMnO₄ exposure or gill mucus swabbing, the gill sections from the KMnO₄ treated fish were compared with the fish in the negative control group.

4.2.3. *Neoparamoeba* spp. isolation and infection

Neoparamoeba spp. were harvested from the gills of AGD-affected Atlantic salmon held in the School of Aquaculture, University of Tasmania (refer to chapter 2, section 2.1 for details on amoebae isolation). Two days post KMnO_4 treatment, fish in groups 1 and 2 were infected with *Neoparamoeba* spp. at a concentration of 300 cells l^{-1} . The control group (Group 3) remained uninfected.

4.2.4 Sampling procedures: Post-amoebae infection

Six fish from all groups were sampled on days 4, 8 and 12 post amoebae challenge. Fish were euthanased as previously described (see section 4.2.2). Entire gills were removed and the left gill arches were placed in sterile filtered seawater to determine amoebae number. *Neoparamoeba* spp. was harvested from the left gill arches using a technique described by Howard and Carson (1995). Gill associated amoebae number was determined using trypan blue exclusion assay (Philips, 1973). Total number of viable amoebae cells per fish was calculated and divided by the natural log of fish mass to account for scaling differences in gill surface area with fish of different mass (Palzenerger and Phola 1992). The second right gill arch from each fish was processed for AGD assessment (refer to chapter 2, section 2.2 for details on AGD diagnosis).

4.2.5 Statistical analysis

Significant differences between groups were assessed as described in chapter 2, section 2.14

4.3 Results

There was a significant reduction in the gill bacterial load in the fish treated with KMnO_4 . The number of CFU in KMnO_4 untreated and control fish ranged from 3.8×10^3 to 6.3×10^3 CFU ml⁻¹ and 4.3×10^3 to 6.8×10^3 CFU ml⁻¹ respectively; whereas those exposed to KMnO_4 contained between 1×10^3 and 2.3×10^3 CFU ml⁻¹ (Figure 4.1). Histological examination showed that gill tissue of KMnO_4 treated fish was normal and healthy 2 days post- KMnO_4 treatment. Both KMnO_4 treated and untreated groups developed typical AGD lesions when infected with *Neoparamoeba* spp.. There was no significant difference in AGD severity between either groups (KMnO_4 treated and untreated), measured in terms of percentage of filaments with lesions, lesion size and the number of amoebae attached to the gill surface on days 4, 8 and 12 (Figures 4.2 and 4.3). In all cases, there were no significant differences between replicates within treatments.

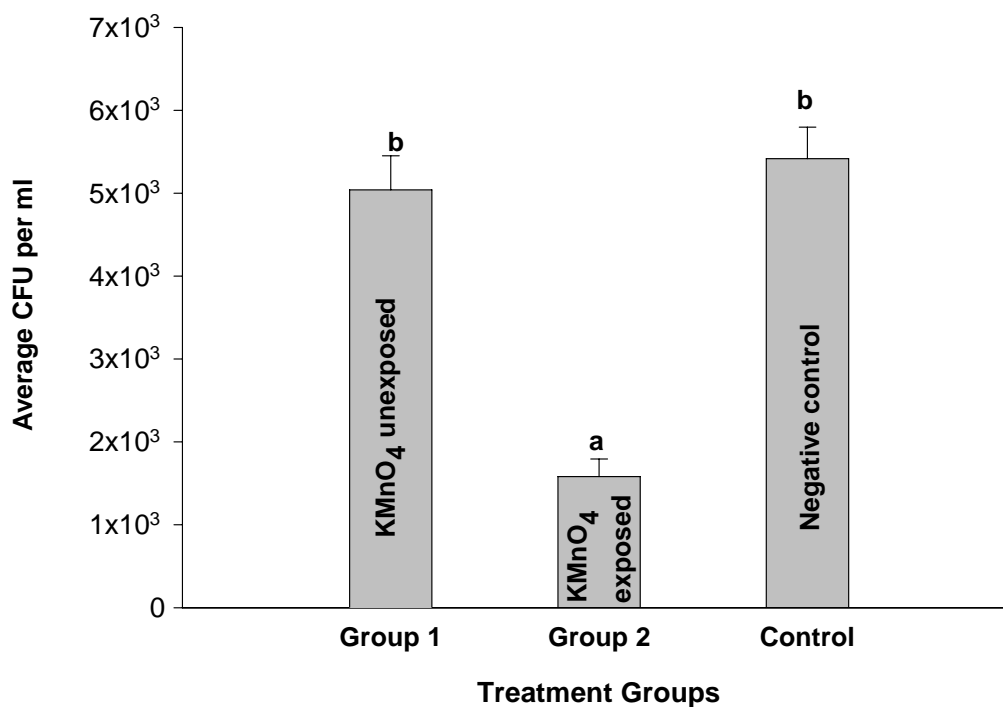


Figure 4.1 The mean \pm SE of the average number of bacterial colonies isolated from salmonid gills in experimental groups (group 1, no KMnO₄ exposure; group 2, exposed to KMnO₄; control, no treatment). Separate analyses were done for each group and means with different letters above the bars represent groups that are statistically different. $n = 6$ fish per group

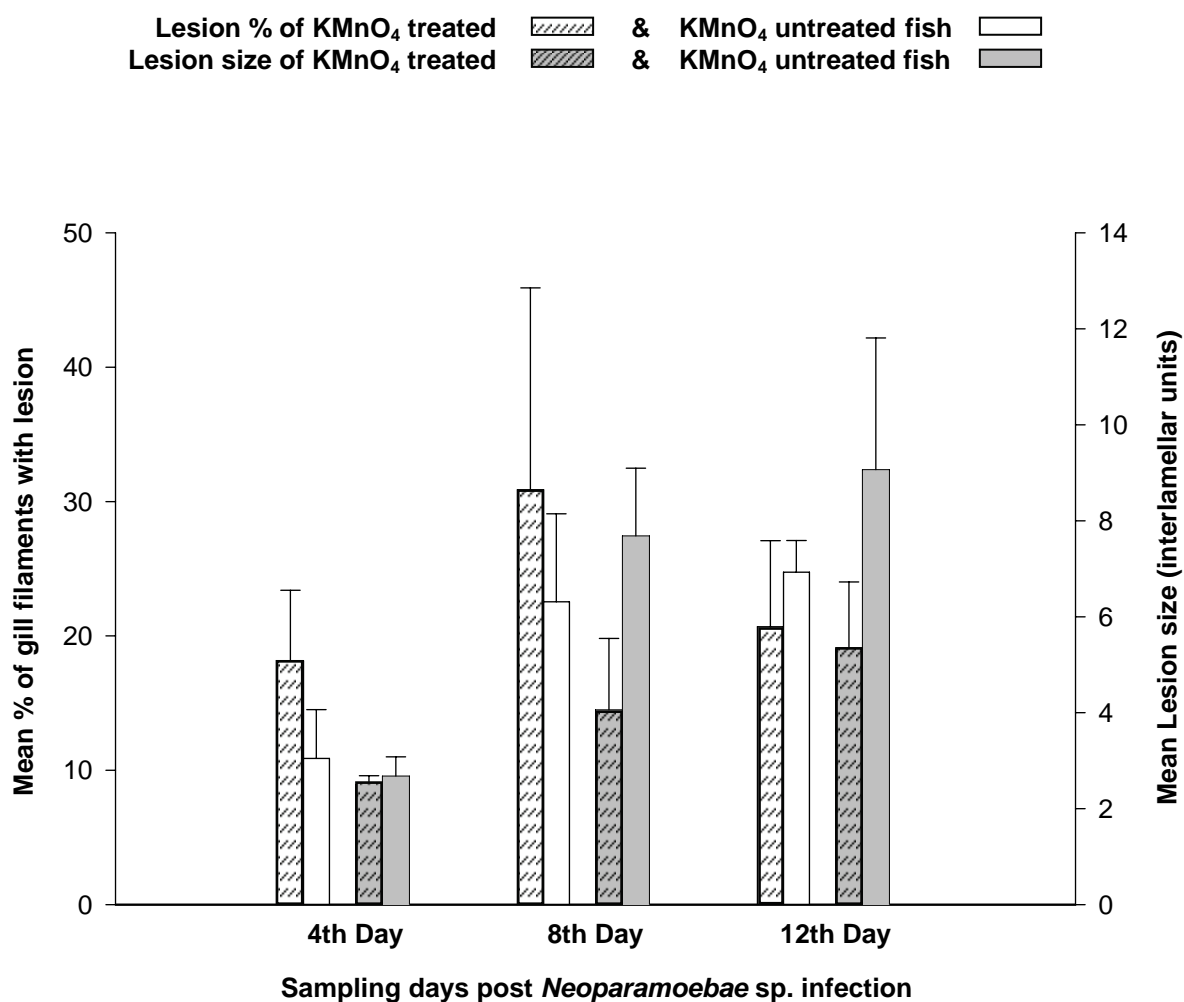


Figure 4.1 The mean \pm SE of percentage of filaments with AGD lesions and number of infected lamellae within in each lesion in KMnO₄ treated and untreated groups. Separate analyses were done for each sampling day and the result was statistically insignificant. $n = 6$ fish per group per sampling day.

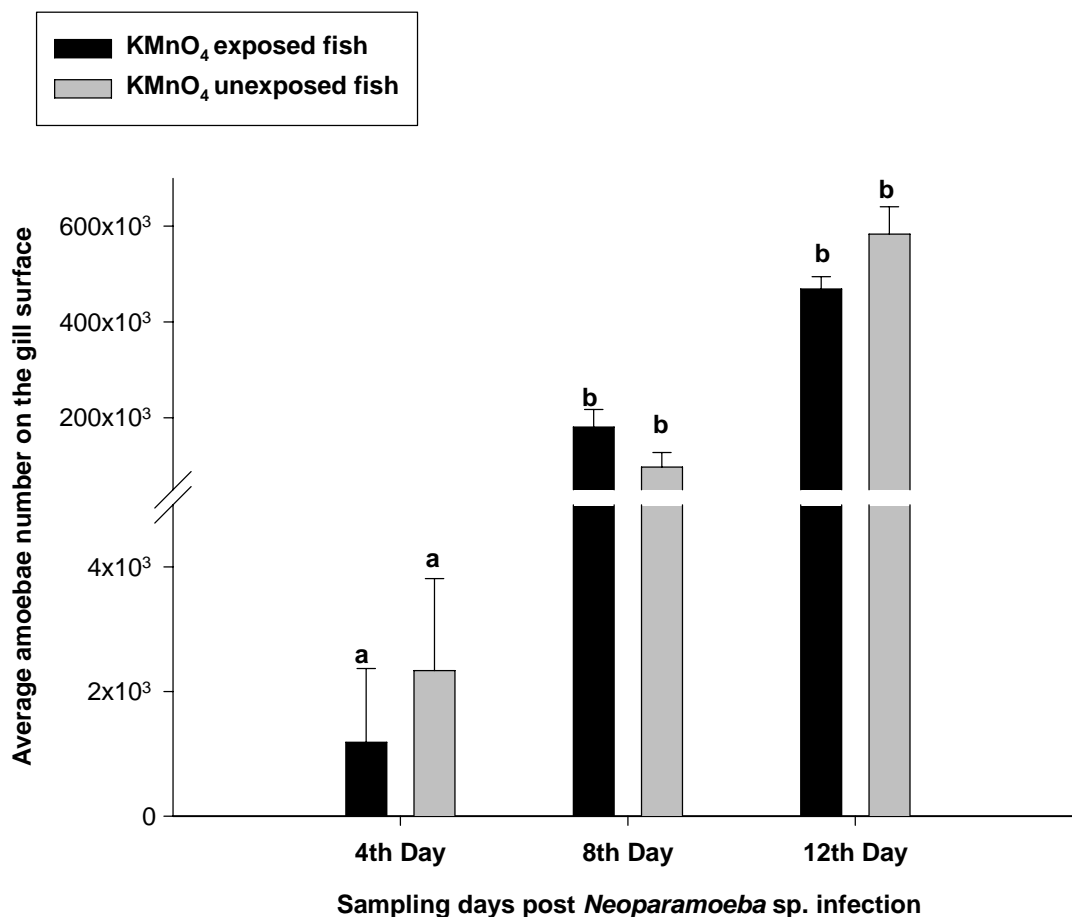


Figure 4.3 The mean \pm SE of average amoebae number on the entire gill surface in KMnO₄ treated and untreated groups. Separate analyses were done for each sampling day and means with similar letters above the bars represent groups that are statistically insignificant. $n = 6$ fish per group per sampling day.

4.4 Discussion

There is extensive information available on use of KMnO_4 for freshwater fish; however, much less information is known about its effect on marine fish. The recommended dosage levels for short term exposure of KMnO_4 as a surface disinfectant for fish in general are 20 mg l^{-1} for 1 h per 50 l of water (Herwig, 1979); 10 mg l^{-1} for 30-60 min (Nowak and Munday, 1992); 10 mg l^{-1} for 30 min (Francis-Floyd and Klinger 2003). However, the reactivity of chemicals in water would vary with salinity and if a chemical is used as a disinfectant or treatment its activity may also vary depending on the target species (Masser and Jensen, 1991). For example, Fathead minnows, *Pimephales promelas* can tolerate 12 mg l^{-1} of KMnO_4 in pond water (Tucker and Boyd, 1977), whereas Lake Malawi cichlids, *Metriaclima zebra* are more sensitive to KMnO_4 with the recommended dose being as low as 1 mg l^{-1} (Francis-Floyd and Klinger, 2003). Hence, to test any adverse effects of a chemical when an effective concentration is unknown, a simple bioassay should be conducted (Masser and Jensen, 1991; Francis-Floyd and Klinger, 2003).

In this study preliminary exposure to a recommended concentration of KMnO_4 of 10 mg l^{-1} for 20 min was detrimental to fish as they moved vigorously and appeared to be restless and stressed. Adjustment to 5 mg l^{-1} of KMnO_4 for 20 min showed no detrimental effect on fish behaviour.

The gills of channel catfish, *Ictalurus punctatus* exposed for 36 h to therapeutic concentration of KMnO_4 (0.438 mg l^{-1}) had mild hypertrophy and

spongiosis in the gills when sampled during exposure; although no lesions were noted 2 days post exposure (Darwish et al. 2002). However, in the current study fish had no signs of pathology due to KMnO_4 exposure. Also, histological analysis conducted two days post KMnO_4 treatment confirmed the gills of fish in each group to be normal and healthy.

Jee and Plumb (1981) reported that KMnO_4 (4 mg l^{-1}) is an effective bactericide for Fathead minnows infected with *Flexibacter columnaris*. Researchers in other fields have reported that washing lettuce samples with KMnO_4 (25 ppm) reduced aerobic microorganisms by more than two log units and total coliforms by at least one log (Soriano et al. 2000). Similarly, fumigation of eggs with formaldehyde (372 g l^{-1}) and KMnO_4 (20 g m^{-3}) eliminated contaminating bacteria from the egg shells (Furuta and Maruyama, 1980). In the current experiment, the number of bacterial colonies on the KMnO_4 treated fish was reduced by approximately $2 \times 10^3 \text{ CFU ml}^{-1}$ compared to untreated fish.

These results indicate that short-term exposure (20 min) of Atlantic salmon to KMnO_4 (5 mg l^{-1}) can reduce gill bacterial load without altering the ability of salmonid to become infected with *Neoparamoeba* spp.

Chapter 5

Influence of salmonid gill bacteria on development and severity of amoebic gill disease

Published in Diseases of Aquatic Organisms 67: 55-60, 2005

Please note: The published paper is slightly modified to suite the thesis format and also new AGD literature is incorporated to make this chapter up to date.

Influence of salmonid gill bacteria on development and severity of amoebic gill disease

5.1 Abstract

The relationship between salmonid gill bacteria and *Neoparamoeba* spp., the aetiological agent of amoebic gill disease (AGD) was determined *in vivo*. Fish were divided into 4 groups and were subjected to following experimental infections: Group 1, amoebae only; Group 2, *Staphylococcus* sp. and amoebae; Group 3, *Winogradskyella* sp. and amoebae; Group 4, no treatment (control). Fish (Groups 1, 2 and 3) were exposed to potassium permanganate to remove/reduce the natural gill microflora prior to either bacterial or amoebae exposure. AGD severity was quantified by histological analysis of gill sections to determine the percentage of lesioned filaments and the number of affected lamellae within each lesion. All amoebae infected groups developed AGD, with fish in Group 3 showing significantly more filaments with lesions than other groups. Typically, lesion size averaged between 2 to 4 interlamellar units in all AGD infected groups. The results suggest that the ability of *Neoparamoeba* spp. to infect filaments and cause lesions might be enhanced in the presence of *Winogradskyella* sp.. The possibility is proposed that the prevalence of more severe AGD is due to the occurrence of *Winogradskyella* sp. at high concentrations on the gills.

5.2 Introduction

Amoebic gill disease (AGD) is one of the most significant health problems confronted by the salmon aquaculture industry in Tasmania (Munday et al. 2001). Even though *Neoparamoeba* spp. is presumed to be the causative agent of AGD (Munday et al. 2001; Dyková et al. 2005), the exact environmental conditions or health status of the fish that allow *Neoparamoeba* spp. to proliferate on fish gills are still unknown and Koch's postulate is yet to be fulfilled for the disease (Howard et al. 1993). Until now AGD infection has always been established by cohabiting naïve fish with infected fish (Howard et al. 1993, Akhlaghi et al. 1996), or by exposing fish to isolated, gill-associated *Neoparamoeba* spp. (Zilberg et al. 2001, Morrison et al. 2004, Morrison et al. 2005) as the disease cannot be reproduced using cultured organisms (Kent et al. 1988, Howard et al. 1993, Findlay et al. 2000, Morrison et al. 2005). According to previous studies, AGD outbreaks may be influenced by factors such as immune status, stocking densities, temperature and salinity (Nowak and Munday 1994, Findlay and Munday 1998, Findlay et al. 2000, Zilberg and Munday 2000, Nowak 2001). Lom and Dyková (1992) also suggest that amphizoic amoebae might typically only colonize the gills of partially immunosuppressed fish where bacterial growth and mucus provide a ready food source. Furthermore, Bowman and Nowak (2004) identified a series of bacteria representing a range of distinct ecotypes from the gills of healthy and AGD infected marine farmed Atlantic salmon. These authors suggested that gill bacteria might play a direct role by predisposing the fish to AGD, to exacerbate AGD, or if bacteria are present in increased numbers in water, might be coincident with AGD outbreaks (Bowman and Nowak 2004). The culture-

dependant study frequently isolated *Winogradskyella* and *Staphylococcus* species bacteria only on AGD-affected fish gills and therefore, suggested that these bacteria might be linked to AGD (chapter 3). The aim of this research is to determine the role of these bacteria (*Winogradskyella* and *Staphylococcus* species) in the incidence and severity of AGD.

5.3 Materials and methods

5.3.1 Experimental tanks and treatment groups

Atlantic salmon *Salmo salar* L. (n = 72; mean weight = 88 g) were acclimatised to sea water (35‰, 1 µm filtered) over a week in 6 identical recirculating systems (refer to chapter 2, section 2.12 for details about recirculating systems setup and seawater parameters). A sentinel population (n = 12) of the same body weight was acclimatised in a static tank (210 l). Following acclimatisation, fish in the recirculating systems were divided into 3 treatment groups (n = 12 fish per treatment i.e. 4 fish per tank). Each treatment was duplicated. The 4th group was the sentinel population (n = 12). Fish in Group 1 were exposed to amoebae only (positive control); Group 2, *Staphylococcus* sp. and amoebae; Group 3, *Winogradskyella* sp. and amoebae; Group 4 did not receive any treatment.

5.3.2. *Neoparamoeba* spp. isolation

Neoparamoeba spp. were harvested from the gills of AGD-affected Atlantic salmon held in the School of Aquaculture, University of Tasmania (refer to chapter 2, section 2.1 for details on amoebae isolation).

5.3.3 Bacteria cultivation

Staphylococcus sp. strain AC8 and *Winogradskyella* sp. strain AC1 bacteria were selected from previously isolated and characterised gill bacteria strains from AGD affected Atlantic salmon from commercial farms in Tasmania (refer to chapter 3 for details on bacteria identification). The same strains are used through out this experiment. *Winogradskyella* sp. bacteria were cultured in Shieh's broth (Song et al. 1988) and *Staphylococcus* sp. bacteria were cultured in Todd Hewitt broth (Oxoid, Australia) as previously described (refer to chapter 2, section 2.7 for details on bacterial cultivation).

5.3.4 Bacteria characterisation

The colony morphology and biochemical profiles (API 50 CH and API Zym, bioMérieux Australia Pty. Ltd.) of *Winogradskyella* sp. and *Staphylococcus* sp. were noted for identification purposes (refer to chapter 2, section 2.5 for details on API tests). Gram strain reactions were conducted based on the methodology described by Hendrickson (1994). The bacterial cells were viewed by a light microscope (Olympus) at 1000 x magnification and the cell length was measured by using a

Leica DC300f digital camera (Wetzlar) attached to the microscope and sigma scan pro, image analysis software version 5.0.0.

5.3.5 Potassium permanganate (KMnO₄) treatment

Prior to inoculation with *Winogradskyella* sp. and *Staphylococcus* sp., fish from groups 1, 2 and 3 were treated with KMnO₄ as previously described (refer to chapter 2, section 2.13 for details about KMnO₄ bathing procedure); this treatment was shown to reduce the gill bacterial levels with no adverse effects to Atlantic salmon (chapter 4). The sentinel fish (Group 4) were handled in the same way but were bathed in a tank containing sea water only. Two days after the KMnO₄ bath, i.e. when the fish returned to normal conditions, 2 fish from each group were euthanised (anaesthetic overdose at 20 ml l⁻¹ Aqui-S[®]). Gill mucus and kidney samples were collected from the euthanised fish and inoculated onto a range of media including Shieh's medium (Song et al. 1988), Marine Agar (Difco), Tryptone Soya Agar (Oxoid), Todd Hewitt (Oxoid) to establish baseline community structure. The agar plates were incubated at 22°C for 24 to 48 h.

5.3.6 Bacteria inoculation

The remaining fish in Groups 2 and 3 were inoculated with *Staphylococcus* sp. and *Winogradskyella* sp. respectively by transferring fish using individual nets into 2 static tanks containing either *Staphylococcus* sp. or *Winogradskyella* sp. bacterium at a density of 1×10^8 cells l⁻¹ and bathed for 1 h. Groups 1 and 4 were handled in the same manner but were bathed in sea water only. After transferring

back to their respective systems, fish were maintained for 4 days to allow the development of inoculated colonies on the gills. All the groups, with the exception of Group 4 were then infected with *Neoparamoeba* spp. at a concentration of 300 cells l⁻¹.

5.3.7 Sampling procedures

The experiment was terminated and fish from all groups sampled on Day 8 post amoebae challenge when white mucous patches (consistent with AGD gross pathology) were observed on the gills of the treatment groups. Fish were euthanised and swabs of gill mucus and anterior kidney were taken and inoculated onto bacterial media as previously described (see section 5.3.5) and incubated at 22°C for 48 h. Colonies were then examined and confirmed as either *Staphylococcus* sp. (Group 2) or *Winogradskyella* sp. (Group 3) by comparing the morphology (shape, pigmentation colour, size and appearance on culture plates) and the biochemical profiles with the previously recorded data. Immediately after swabbing for bacteriology, all gills were removed for AGD assessment (refer to chapter 2, section 2.2 for details on AGD diagnosis).

5.3.8 Statistical analysis

Significant differences between groups were assessed as described in chapter 2, section 2.14.

5.4 Results

All groups infected with gill amoebae showed gross gill lesions by Day 8 of the experiment. The negative control (Group 4) did not have gill pathology consistent with AGD at any time during the experiment.

5.4.1 Histopathology

All amoebae infected fish (Groups 1, 2 and 3) had AGD. However, fish exposed to *Winogradskyella* sp. (Group 3) had significantly more filaments (51%) with lesions than the other groups ($F = 21.9$, $df = 3, 73$, $p < 0.001$; Figure 5.1). The majority of histological sections in this group showed the presence of large numbers of *Winogradskyella* sp. bacterium on the filaments (Figure 5.2A) whereas in Group 2, (pre-exposed to *Staphylococcus* sp.) only small numbers of bacteria were observed. There was no difference in percentage of affected filaments in Group 2 compared to Group 1 (no bacteria); both groups of fish had approximately 16% of gill filaments with lesions. Despite the significant increase in the percentage of lesions on each filament in Group 3, there was no difference in the size of lesions (interlamellar units) between treatment groups. Typically, lesions size averaged between 2 to 4 interlamellar units (Figure 5.1). In addition, there were no apparent differences in lesion structure. A thorough investigation of the gill sections revealed that all the treatment groups had severe lesions consisting of completely fused secondary lamellae and an almost continuous layer of amoebae was observed on the surface of hypertrophic tissue. Lesions were typified by epithelial desquamation, filamental and lamellar oedema, interlamellar vesicles containing amoebae, infiltration of the central

venous sinus with leucocytes and severe hyperplasia of the epithelium (Figure 5.2B,C). The control group had healthy gills free from AGD infection. In all cases, there were no significant differences between replicates within treatments.

5.4.2 Bacterial recovery

No bacterial growth was detected on the culture plates from the gill and kidney swabs collected from fish 2 d post-KMnO₄ bath. At Day 8 post-amoebae infection gill bacteria were very low in number, absent or non-culturable from the gills of Groups 1 and 4 and from the kidney of all the groups. Inoculated bacteria were recovered from the fish gills in Groups 2 and 3 on Day 8 post-amoebae infection. In each case the majority of the recovered colonies were of a single species with morphological and biochemical characteristics consistent with previously recorded data of *Staphylococcus* sp. (Group 2) and *Winogradskyella* sp. (Group 3). Briefly, *Winogradskyella* sp. were Gram negative, rod shaped cells, with a cell length of $\approx 0.86 \mu\text{m}$ and cell width $\approx 0.39 \mu\text{m}$. The colonies were yellow pigmented, entire and translucent with low convex elevation and 2 mm in length. The results from API tests showed that *Winogradskyella* sp. metabolised alkaline phosphatase, esterase lipase (C8), α -chymotrypsin, leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS BI phosphohydrolase and formed acid with glycerol, L-arabinose, ribose, D-xylose, galactose, glucose, mannose, manitol, sorbitol, melibiose, sucrose, trehalose, D-fucose, D-arabitol, L-arabitol and Gluconate. Likewise, *Staphylococcus* sp. were Gram positive, coccus shaped cells arranged in clusters. The cell length was $\approx 0.9 \mu\text{m}$ and width was $\approx 0.39 \mu\text{m}$. The colonies were

yellow pigmented, entire and opaque with low convex elevation and 2 mm in length.

Staphylococcus sp. formed acid with glucose, fructose, manitol, maltose and sucrose.

It also metabolised esterase (C4) and naphthol-AS BI phosphohydrolase.

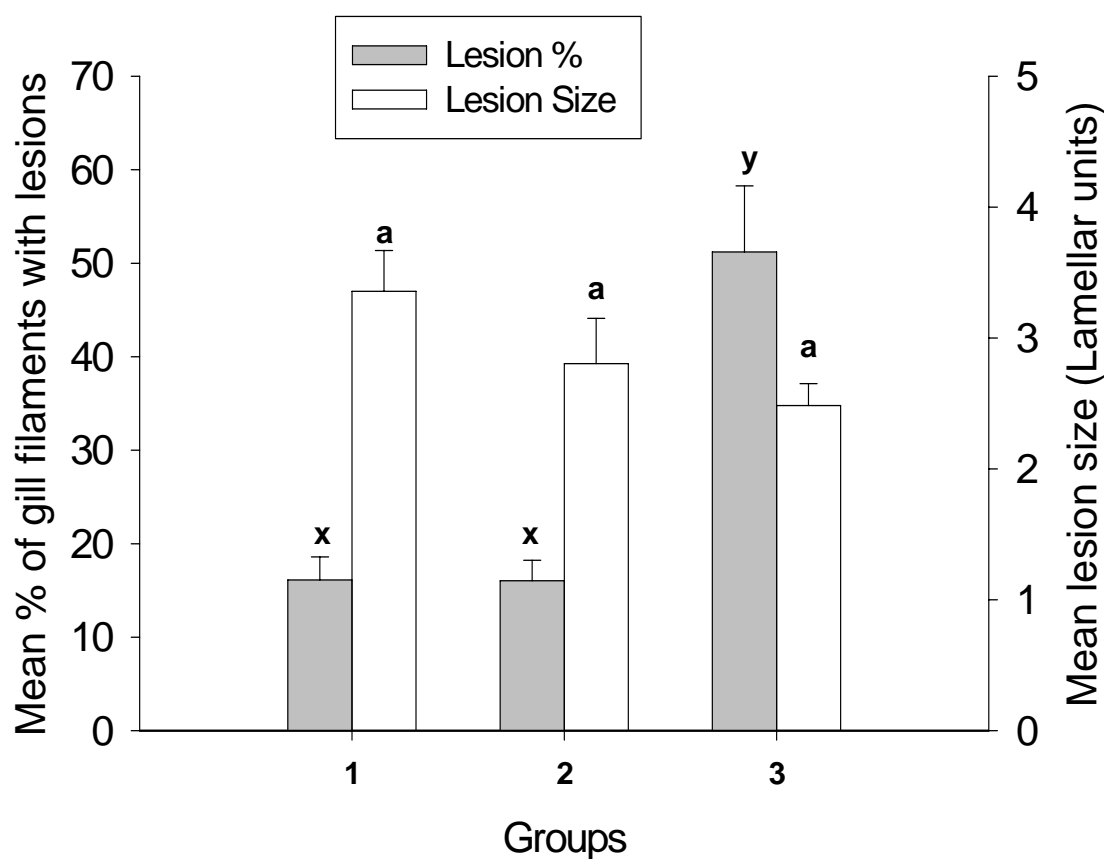


Figure 5.1 *Salmo salar*. The mean \pm SE of percentage of filaments with lesions and number of infected lamellae within in each lesion in experimental groups: (Group 1, *Neoparamoeba* spp.; Group 2, *Staphylococcus* sp. and *Neoparamoeba* spp.; Group 3, *Winogradskyella* sp. and *Neoparamoeba* spp.). Separate analyses were done for lesion percentage and lesion size (lamellar units). Different letters above error bars indicate groups are significantly different. $n = 22$ for all treatment groups

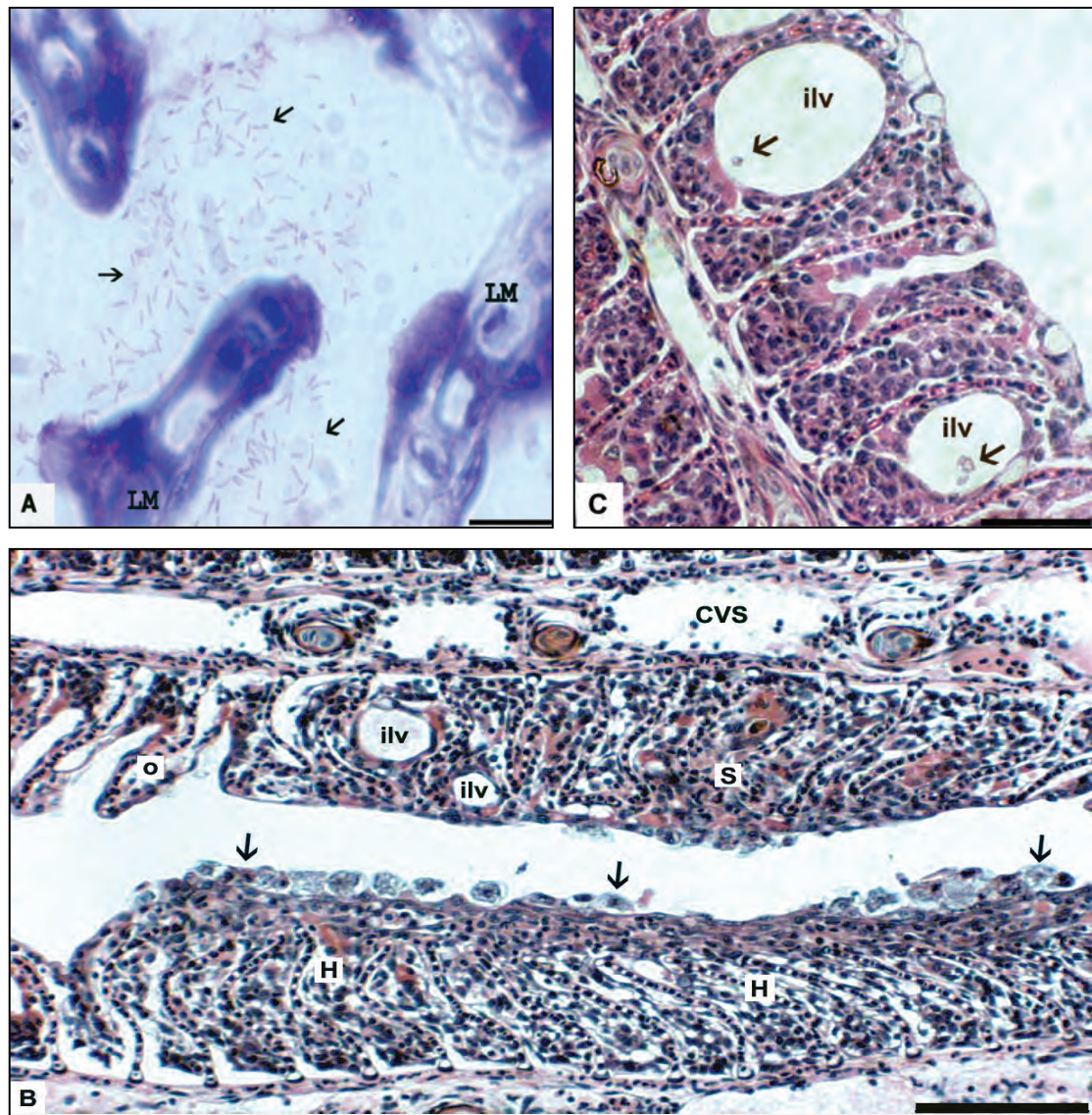


Figure 5.1 *Salmo salar*. (A) Gram stained gill of AGD-infected salmon. *Winogradskyella* sp. (arrows) were present in large numbers on the surface of the lamellae (LM) and were free within the interlamellar space. Scale bar = 10 μ m. (B) Severely infected gill consisting of a continuous row of *Neoparamoeba* sp. (black arrows) on the surface of hyperplastic interlamellar units (H). Oedematous (O) gill tissue showing epithelial spongiosis (S), interlamellar vesicles (ilv) and infiltration of the central venous sinus with leucocytes (cvs). Scale bar = 100 μ m. (C) AGD affected gill showing interlamellar vesicles (ilv) containing *Neoparamoeba* sp. (arrows). Scale bar = 50 μ m

5.5 Discussion

Roubal et al. (1989) were the first to suggest a role for gill colonising bacteria in AGD and considered that management strategies aimed at reducing bacterial levels could in turn reduce AGD. Similarly Bowman and Nowak (2004) provided discussion on the presence of *Neoparamoeba* spp. and high bacterial populations that may lead to more pronounced incidence of AGD. The present study is the first experimental attempt to determine a relationship between some gill-associated bacteria and AGD.

This study showed an apparent involvement of the Gram negative bacterium *Winogradskyella* sp. in AGD. Fish experimentally infected with this bacterium showed increased numbers of gill lesions following *Neoparamoeba* spp. infection, whilst those exposed to Gram positive *Staphylococcus* sp. did not show gill pathology that was different from the positive control (Group 1). Positive control animals were previously treated with KMnO₄ and gill bacteria were very low in number, absent or non-culturable during subsequent AGD infection. These fish did however develop the disease at a rate comparable to those infected with *Staphylococcus* sp. and consistent with the typical pattern of AGD initiated by experimental infection. All fish for this study were from the same source and same handling procedures were followed for all groups during the entire period of the experiment except for the treatment. Therefore, despite the fact that the gill bacterial status at the beginning of the infection is unknown, the difference in AGD lesion

severity between the treatment groups is exclusively due to experimental addition of bacteria.

Roubal et al. (1989) observed the association of bacteria with amoebae during infection of salmon gills. Similarly, Douglas-Helders et al. (2003) reported a co-existing *Flavobacterium* infection on AGD infected salmon gills. Bowman and Nowak (2004) showed a higher proportion of Gram negative bacteria on the gills of marine farmed AGD infected Atlantic salmon. In the present study, a high percentage of hyperplastic lesions on each filament were noted on the gills in the presence of the Gram negative bacterium *Winogradskyella* sp.. Phylogenetic analysis of 16S rRNA sequences revealed the bacteria from the genus *Winogradskyella* are closely related to *Psychroserpens burtonensis* with a 93.5 to 93.8% similarity (Nedashkovskaya et al. 2004). Further corroborating evidence by Bowman and Nowak (2004) indicated that a *Psychroserpens* sp. phylotype dominated the bacterial community in AGD infected salmon gill samples.

The nourishment for *Neoparamoeba* spp. survival and growth, once it has gained access to salmon gills, has not been clearly identified. Previous researchers suggest that other infectious amoebae may feed on gill bacteria (Noble et al. 1997) and perhaps attain bloom populations in the presence of abundant food organisms (Kent et al. 1988). *In vitro* growth of trophozoites of a *Platyamoeba* strain isolated from the diseased gill tissues of cultured turbot increased considerably in the presence of *Aeromonas hydrophila*, *Vibrio natriegens*, *Pseudomonas nautica* and *Escherichia coli* (Paniagua et al. 2001). Similarly, the number of *Acanthamoeba*

castellanii and *A. polyphaga* were enhanced by cocultivation with the Gram negative bacteria, *Xanthomonas maltophilia*, *Flavobacterium breve* and *Pseudomonas paucimobilis* (Bottone et al. 1992). Based on the above findings, it is tempting to suggest that bacteria might provide a food source to *Neoparamoeba* spp. during initial stages of colonisation and infection. However, recent research on AGD has suggested that *Neoparamoeba* spp. isolated from AGD infected fish gills are not bacterivorous (Dyková and Lom 2004). Despite this, during the present study the fish inoculated with *Winogradskyella* sp. and amoebae showed an increased number of gill lesions. Therefore, some interaction exists between bacteria and amoebae, which needs to be characterised. Bracha and Mirelman (1984) reported that co-incubation of *Entamoeba histolytica* with various types of Gram-negative bacteria increased the virulence and ability of amoebae to destroy monolayers of baby hamster kidney cultured cells. Therefore, one explanation for the increased number of AGD lesions noted in this study might be the presence of *Winogradskyella* sp. on the gills which possibly enhanced the ability of *Neoparamoeba* spp. to infect filaments and cause AGD lesions.

The current study also showed that *Neoparamoeba* spp. can infect gills and cause AGD in salmonids (Group 1) after KMnO₄ disinfection and with very low levels of culturable bacteria. Therefore, in agreement with other authors we have shown that the amoeba can be a primary pathogen and cause AGD in salmon (Kent et al. 1988, Roubal et al. 1989, Munday et al. 1990, Dyková et al. 1995, Zilberg and Munday 2000, Adams and Nowak 2003, 2004).

We propose that the ability of *Neoparamoeba* spp. to infect filaments and cause lesions might be enhanced in the presence of *Winogradskyella* sp. strain AC1. However, further research is needed to determine whether an increase in the concentration of *Winogradskyella* sp. on the gills increases the rate of incidence and severity of AGD. The effect of *Winogradskyella* sp. alone on the fish gills should also be determined. In addition, it is essential to find the effect of other gill-colonising organisms on AGD. If bacteria are found to influence the progression and severity of AGD then the knowledge may be useful for designing alternative control strategies.

Chapter 6

Concentration effects of *Winogradskyella* sp.
in the incidence and severity of amoebic gill
disease

Published in Diseases of Aquatic Organisms 73: 43-47, 2006

6

Concentration effects of *Winogradskyella* sp. in the incidence and severity of amoebic gill disease

6.1 Abstract

To study the concentration effects of *Winogradskyella* sp. bacterium on amoebic gill disease (AGD), Atlantic salmon (*Salmo salar*) were pre-exposed to two different doses (10^8 or 10^{10} cells l^{-1}) of *Winogradskyella* sp. before challenging with *Neoparamoeba* spp.. Exposure of fish to *Winogradskyella* sp. caused a significant increase in the percentage of AGD affected filaments compared to controls challenged with *Neoparamoeba* only; however these percentages did not increase significantly with an increase in bacterial concentration. The results show that the presence of *Winogradskyella* sp. on salmonid gills can increase the severity of AGD.

6.2 Introduction

Amoebic gill disease (AGD) in Atlantic salmon, *Salmo salar* L. is one of the significant problems faced by the south eastern aquaculture industries in Tasmania. The causative agent of AGD is *Neoparamoeba* spp. (reviewed by Munday et al. 2001; Dyková et al. 2005). Although extensive research has been conducted to study either bacterial (Sawyer 1978; Rintamaeki and Valtonen 1991; Cipriano et al. 1992; Ford et al. 1998; Valheim et al. 2000) or amoebic disease in Atlantic salmon (reviewed by Munday et al. 2001; Adams and Nowak 2004; Dyková et al. 2005) only a few studies have attempted to examine the association between salmonid gill

bacteria and AGD. Bowman and Nowak (2004) assessed the bacterial communities of salmon gills using culture-independent 16S ribosomal RNA analyses and suggested that the bacteria from the genus *Psychroserpens* which were only detected on AGD-affected fish gills, might be linked to AGD. A recent 16S rRNA gene-based study, identifying the culturable gill bacteria in AGD-affected salmonids showed that the gills were predominantly colonised by bacteria that had biochemical profiles similar to *Winogradskyella* spp. (clusters AC1, AC2 and BC1; chapter 3). In addition, recent experiments conducted to study the influence of bacteria in the development and severity of AGD showed that fish experimentally infected with *Winogradskyella* sp. strain AC1 developed increased numbers of gill lesions following *Neoparamoeba* spp. infection (chapter 5). Therefore, the current experiment aims to study the concentration effects of this bacterium on AGD.

6.3 Materials and methods

6.3.1 Experimental setup

Atlantic salmon *Salmo salar* L. (n = 72; mean weight = 115 g) were acclimatised to seawater (30‰, 2 µm filtered) over two weeks in 4000 l tanks connected to individual biofilters. Subsequently, fish were treated with potassium permanganate, KMnO₄ to reduced natural microflora on the gills (refer to chapter 2, section 2.13 for details about KMnO₄ bathing procedure); this treatment was shown to reduce the gill bacterial levels with no adverse effects to Atlantic salmon (chapter 4). Following KMnO₄ treatment, fish were transferred to 6 identical recirculating systems for further acclimation to salinity 35‰ over a period of 2 days (refer to

chapter 2, section 2.12 for details about recirculating systems setup and seawater parameters).

6.3.2 Treatment groups

Following acclimatisation, fish in the recirculating systems were divided into 3 treatment groups (n = 12 fish per treatment i.e. 4 fish per tank). Each treatment was duplicated. Fish in group 1 were exposed to *Neoparamoeba* spp. only (positive control); group 2, *Winogradskyella* sp. (10^8 cells l^{-1}) and *Neoparamoeba* spp.; group 3, *Winogradskyella* sp. (10^{10} cells l^{-1}) and *Neoparamoeba* spp..

6.3.3 *Neoparamoeba* spp. isolation

Neoparamoeba spp. were harvested from the gills of AGD-affected Atlantic salmon held in the School of Aquaculture, University of Tasmania (refer to chapter 2, section 2.1 for details on amoebae isolation).

6.3.4 Bacteria cultivation and characterisation

Cryopreserved samples (refer to chapter 2, section 2.4 for details on cryopreservation of bacteria) of *Winogradskyella* sp. strain AC1 previously isolated from AGD-affected fish gills (refer to chapter 3 for details on bacteria identification) were cultured in Shieh's broth (Song et al. 1988) (refer to chapter 2, section 2.7 for details on bacterial cultivation). For identification purposes, the cells were grown on Shieh's agar (Song et al. 1988) and the colony morphology and enzymatic activity of *Winogradskyella* sp. strain AC1 were recorded (refer to chapter 2, section 2.5 for details on API Zym test procedure). Gram stain reactions were conducted based on

the methodology described by Hendrickson (1994). The bacterial cells were viewed by a light microscope (Olympus) at 1000 x magnification and the cell length was measured by using a Leica DC300f digital camera (Wetzlar) attached to the microscope and sigma scan pro, image analysis software version 5.0.0.

6.3.5 Bacteria and amoebae exposure

Fish in groups 2 and 3 were inoculated with *Winogradskyella* sp. by means of a bath treatment for 1 h. Briefly, fish were transferred using individual nets from the recirculating systems into 2 static tanks filled with seawater (salinity 35‰) and containing bacteria at concentrations of 10^8 cells l^{-1} and 10^{10} cells l^{-1} respectively. Group 1 fish were handled in the same manner but were bathed in seawater only. All fish were returned to their respective systems and were maintained for 4 days to allow the development of inoculated colonies on the gills. All the groups were then infected with *Neoparamoeba* spp. at a concentration of 300 cells l^{-1} .

6.3.6 Day 0 sampling

Immediately prior to *Neoparamoeba* spp. inoculation a sub-sample of 6 fish from each group were euthanased by Aqui-S[®] (20 ml l^{-1}) and the second left gill arch and anterior kidney were gently swabbed and inoculated onto Shieh's agar (Song et al. 1988) and incubated at 22°C for 48 h. After swabbing for bacteriology, all gills were removed and placed in seawater Davidson's fixative (Shaw and Battle 1957) for 12 h then post-fixed in 70% ethanol to assess gill damage possibly caused by *Winogradskyella* sp. exposure. The second right gill arch was wax embedded, 5 µm

sections were cut and Gram stained. All sections were viewed under light microscope at magnifications ranging from X10 to X1000 (Olympus, Germany).

6.3.7 Day 8 sampling

The experiment was terminated and fish from all groups were sampled on day 8 post amoebae challenge. Fish were euthanased and gill swabs were collected for bacteriology and histology as described above (section 6.3.6). To diagnose AGD, 5 µm section of second right gill arch was stained with haematoxylin and eosin (refer to chapter 2, section 2.2 for details on AGD assessment).

6.3.8 *Winogradskyella* sp. retrieval

To confirm the presence of *Winogradskyella* sp. strain AC1, culture plates were examined and the colonies that had similar morphology (i.e. colonies are yellow pigmented, entire and translucent with low convex elevation and 2 mm in length), Gram characteristics (i.e. Gram negative rod shaped cells) and size (i.e. cell length of ≈ 0.86 µm and cell width ≈ 0.39 µm) to *Winogradskyella* sp. strain AC1 were selected and API Zym profiles (i.e. ability to metabolise alkaline phosphatase, esterase lipase (C8), α -chymotrypsin, leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS BI phosphohydrolase) matched with those of *Winogradskyella* sp. strain AC1.

6.3.9 Statistical analysis

Significant differences between groups were assessed as described in chapter 2, section 2.14.

6.4 Results

6.4.1 Bacteriology

Winogradskyella sp. strain AC1 was successfully retrieved from all fish in bacteria treated groups (group 2, exposed to *Winogradskyella* sp. at 10^8 cells l^{-1} and group 3, exposed to 10^{10} cells l^{-1} prior to amoebae challenge) on both sampling days i.e. on day 0 (before *Neoparamoeba* spp. exposure) and day 8 (8 days post *Neoparamoeba* spp. exposure). In addition, fish from both these groups had clusters of bacteria in their interlamellar space, which could be seen in histological sections (Figure 6.1a). Despite the fact that some bacteria were isolated from group 1, which was not exposed to *Winogradskyella* sp., the colony morphology, cell shape and size and enzymatic profiles of these bacteria were not consistent with those of *Winogradskyella* sp. strain AC1. None of the fish exposed to *Winogradskyella* sp. had signs of pathology prior to AGD as histological examination conducted four days post exposure to the bacterium confirmed the gills of group 2 and 3 fish to be normal and healthy. No bacterial growth was detected on the culture plates from the kidney swabs collected from groups 1, 2 and 3 on all sampling days.

6.4.2 Assessment of AGD

Histological examination of gill sections showed that the fish in groups 1, 2 and 3 displayed typical AGD lesions characterised by diffusion of primary and secondary lamellae often containing *Neoparamoebae* on the surface of hyperplastic lamellae and presence of interlamellar vesicles, multifocal necrosis and mononuclear

cell infiltration (Figure 6.1b). There were no apparent differences in AGD lesion structure between the three treatment groups. A significant difference in the percentage of filaments with lesions was noted between the fish previously exposed to *Winogradskyella* sp. (group 2 and 3) compared to the fish with no *Winogradskyella* sp. exposure (group 1) prior to *Neoparamoeba* spp. infection ($F = 10.6$, $df\ 2, 51$, $p < 0.001$; Figure 6.2). However, there was no significant difference between the percentage of affected filaments in groups that were exposed to *Winogradskyella* sp. at concentrations $10^8\ \text{cells l}^{-1}$ and $10^{10}\ \text{cells l}^{-1}$ (Figure 6.2); group 2 had approximately 21% and group 3 had 17% of gill filaments with lesions. In addition, there was no significant difference in the lesion size between all groups (1, 2 and 3); the lesion size averaged from 18 to 24 interlamellar units (Figure 6.2). In all cases, there were no significant differences between replicates within treatments.

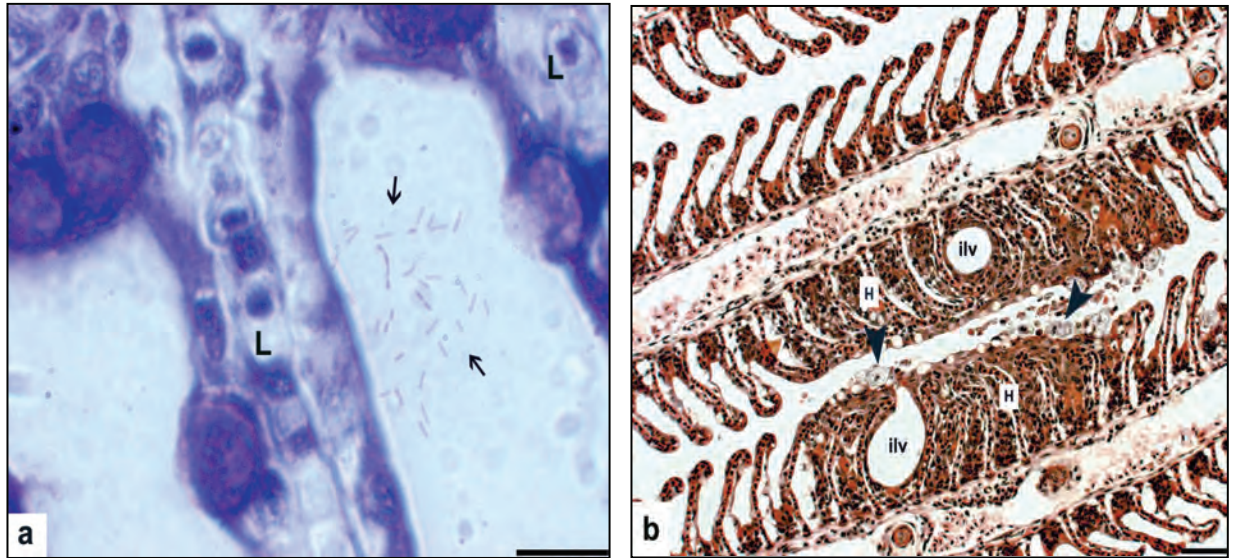


Figure 6.1 (a) Gill section of AGD-affected Atlantic salmon. *Winogradskyella* sp. (arrows) appears scattered in the interlamellar space adjacent to gill lamellae (L). Scale bar = 10 μ m. (b) Gill section showing severe AGD infection characterised by a row of amoebae (arrow heads) on the hyperplastic lamellar (H) surface and occurrence of interlamellar vesicles (ilv). Scale bar = 50 μ m.

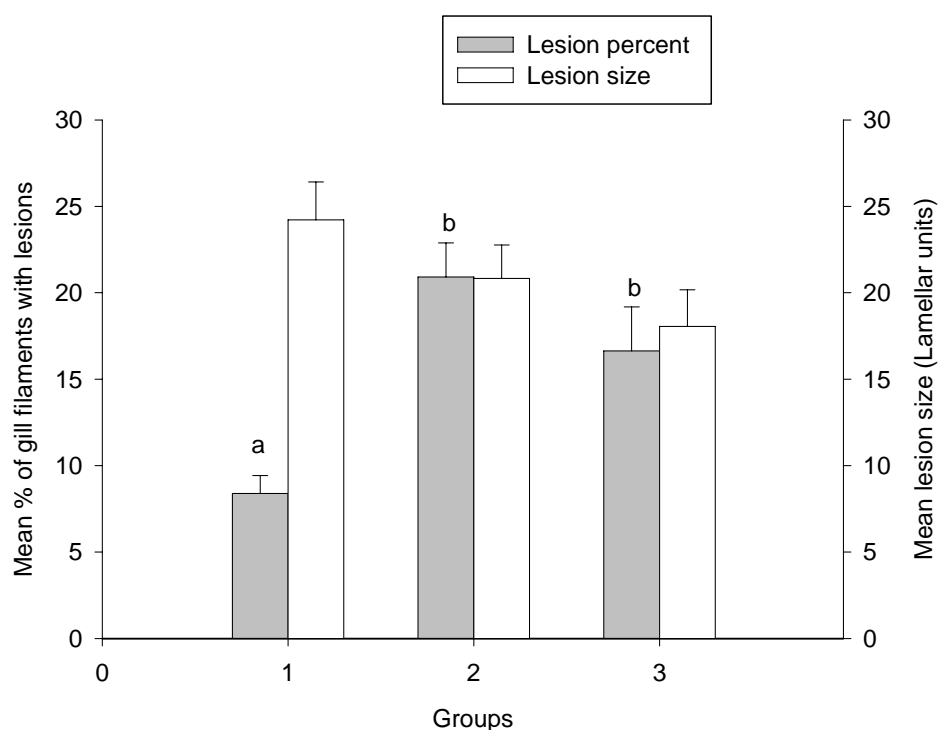


Figure 6.2 The mean \pm SE of percentage of filaments with lesions and number of infected lamellae within in each lesion in treatment groups: (Group 1, *Neoparamoeba* sp.; Group 2, *Winogradskyella* sp. (10^8 cells l^{-1}) & *Neoparamoeba* sp.; Group 3, *Winogradskyella* sp. (10^{10} cells l^{-1}) & *Neoparamoeba* sp.). Separate analyses were done for lesion percentage and lesion size. Different letters above error bars indicate groups are significantly different.

6.5 Discussion

This study demonstrates that fish previously exposed to *Winogradskyella* sp. at concentrations 10^8 cells l^{-1} and 10^{10} cells l^{-1} prior to *Neoparamoeba* spp. infection had significantly higher percentage of filaments with lesions compared to the fish with no *Winogradskyella* sp. exposure. Similarly our previous study (chapter 5) recorded a significant increase in the percentage of affected filaments in the fish that were inoculated with 10^8 cells l^{-1} of *Winogradskyella* sp. prior to amoebae exposure compared to fish with no *Winogradskyella* sp. inoculation. Furthermore, both studies showed no significant difference in the lesion size (number of interlamellar units within each lesion) between the treatment groups. However, in spite of these similarities, the percentage of affected filaments and the lesion sizes were not the same in both experiments. In the current experiment the group that was exposed to 10^8 cells l^{-1} of *Winogradskyella* sp. had approximately 21% of the filaments with AGD lesions compared to the previous study where the number of AGD affected filaments was approximately 51% in the fish exposed to the same concentration of *Winogradskyella* sp. (chapter 5). At the same time, the AGD lesion size averaged between 2 to 4 interlamellar units in the previous study (chapter 5), while it averaged between 18 to 24 interlamellar units in the current study. Thus, even though we noted a similar trend, the numbers were not identical. We suppose that the reasons for these differences might be due to dissimilarity in fish population, since the previous experiment (chapter 5) was conducted with winter smolts while the current experiment was conducted with summer smolts; furthermore, due to unavailability of fish from the same farm, the smolts for the current experiment were procured from a

different farm. In addition, the disparity might also be due to difference in amoebae isolates; because variation in AGD pathology induced by amoebae isolated at different times has been noted previously (unpublished data).

There was no significant difference in the percentage of AGD affected filaments between fish treated with either concentration of *Winogradskyella* sp. prior to amoebae infection. A similar lack of dose-related response was reported in a study involving experimental induction of bacterial kidney disease in Chinook salmon by immersing the fish in various concentrations of *Renibacterium salmoninarum* cells (10^4 to 10^6 ml⁻¹) (Murray et al. 1992). We did not determine whether there were more *Winogradskyella* on 10^{10} group than on the 10^8 group at 4 days post inoculation, we have assumed it to be different and therefore hypothesised four possible reasons for the lack of a dose-related response: firstly, the difference in bacterial concentration ranges (which is 100 times) selected for the bath exposure was probably not big enough to cause a noticeable difference in the severity of AGD; secondly, the increase in the concentration of *Winogradskyella* in group 3 might have increased the number of *Winogradskyella* on the gills and this perhaps restricted the gill surface area required for amoebae colonisation; thirdly, maybe a limit is applicable to the rate of progression of AGD further to which the disease cannot develop any faster and fourthly, it is possible that 4 days after inoculation, the difference in *Winogradskyella* sp. numbers is much less substantial (i.e. proliferation of the lower population and growth limits on the higher) than at the time of inoculation.

In conclusion, this study found that the severity of AGD does not increase with an increase in concentration of *Winogradskyella* sp. i.e. exposure of Atlantic salmon to either 10^8 or 10^{10} cells l^{-1} do not cause a significant difference in the percentage of AGD-affected filaments. Nevertheless, this study reconfirmed that the presence of *Winogradskyella* sp., strain AC1 on the salmonid gills may result in more severe AGD. This suggests that *Winogradskyella* sp. has some effect on Atlantic salmon and AGD. Meanwhile, this study recorded that *Winogradskyella* sp. strain AC1 does not cause any pathology in Atlantic salmon during the first 4 days of its exposure. However, we do not know the pathogenicity of this strain beyond these 4 days, as this experiment did not contain a group that was exposed to *Winogradskyella* alone. Furthermore, the physiological effect of this bacterium on Atlantic salmon is unknown. Therefore, further research is required to examine the pathogenic effect of *Winogradskyella* sp. on Atlantic salmon over a prolonged incubation period. In addition, it is also important to study the influence of *Winogradskyella* sp. on the physiological condition of Atlantic salmon.

Chapter 7

Taxonomy of culturable bacteria isolated over time from marine farmed Atlantic salmon gills during a typical occurrence of amoebic gill disease

7

Taxonomy of culturable bacteria isolated over time from marine farmed Atlantic salmon gills during a typical occurrence of amoebic gill disease

7.1 Abstract

Previous research determining the influence of bacteria in amoebic gill disease (AGD) reported that the presence of *Winogradskyella* sp. on the gills during the process of AGD infection could increase AGD severity (chapters 5 and 6). Therefore, this study examines the diversity and community structure of culturable gill bacteria of seawater reared Atlantic salmon during the typical occurrence of AGD and verifies whether bacteria such as *Winogradskyella* spp. that could exacerbate AGD conditions, frequently colonise the gills. In addition, this study attempts to relate any changes in the gill bacterial community to freshwater treatments and AGD status of fish. Bacteria samples were collected from salmonid gills on five different occasions over a period of 102 days. The cultured bacteria were identified using 16S ribosomal RNA gene analysis. The results showed that *Winogradskyella* spp. might not be consistently present on AGD-affected fish gills. Bacteria closely related to *Pseudoalteromonas* species were the most commonly isolated species; the other more frequently isolated species included *Planococcus*, *Alphaproteobacterium*, *Bizionia* and *Cobetia*. The changes occurring to the gill bacterial community were complex and not easily predictable and several factors including freshwater treatments, fluctuations in salinity and temperature, AGD severity might have influenced these changes.

7.2 Introduction

In Tasmania, amoebic gill disease (AGD) is generally associated with salmonids, most notably Atlantic salmon, *Salmo salar*. The causative agent of AGD is *Neoparamoeba* spp. (reviewed by Munday et al. 2001; Dyková et al. 2005). Approximately 10% of the production cost is spent on AGD treatment, which includes exposing the fish to freshwater for 2 to 3 h. Previous AGD research has reported that freshwater bathing does not cause serious side effects (Powell et al. 2001). However, complete removal of the parasite is not achieved and under favourable conditions, AGD can reoccur within 10 days (Parsons et al. 2001; Clark et al. 2003). Many factors seem to favour invasion by amoebae and among them salinity and temperature are the most important environmental factors influencing the prevalence of AGD (Munday et al. 1990; Clark and Nowak 1999; Douglas-Helders et al. 2001). Roubal et al. (1989) were the first to suggest a role for gill colonising bacteria in AGD. Likewise, several studies have reported the interactions between human pathogenic amoebae and bacteria (Bracha and Mirelman 1984; Bottone et al. 1992; Brieland et al. 1997; Fritsche et al. 2000). Previously a culture-independent study examining the relationship between salmonid gill bacteria and AGD, detected the phylotypes from the genus *Psychroserpens* to be predominantly present on the gills of AGD-affected fish (Bowman and Nowak 2004). A similar study using the culture-dependent techniques frequently isolated bacteria that had biochemical profiles similar to the representative strains identified as *Winogradskyella* sp. from AGD positive fish gills (chapter 3). Furthermore, the *in vivo* trials determining the influence of bacteria in AGD reported that the presence of *Winogradskyella* sp. strain

AC1 during the development of AGD could increase the disease severity (chapters 5 and 6). Based on the above results, it is important to verify whether bacteria such as *Winogradskyella* spp. that could exacerbate AGD conditions frequently colonise the gills of AGD-affected fish present on farms. Therefore, the current study was conducted over a period of time (102 days) and samples were collected on five different occasions from marine farmed Atlantic salmon gills during a typical occurrence of AGD aiming to get a snapshot of the bacteria that regularly colonise salmonid gills. In addition, this study will examine whether the changes occurring in the gill bacterial population is related to the bathing events and AGD status of fish.

7.3 Materials and methods

7.3.1 Fish

Fish were housed in a 5x5x5 m net-pen (125m³ volume) located at Aquatas Pty, Ltd, Tasmania, Australia at a stocking density of 2.8 kg m⁻³ (500 fish per pen; for fish size refer to Table 7.1). The seawater temperature at the farm site was continuously recorded and the mean water temperature 14 days preceding the sampling days is shown in Table 7.1. During the experimental period, fish were exposed twice to freshwater for a period of 2-3 h, a method to control AGD. Cages of fish were towed from their grow-out location to a bathing site. Freshwater was obtained from a dam situated near the farm. The fish were transferred by dip nets from their holding cage to a tarpaulin bathing liner containing up to 9000 l of freshwater (stocking densities of 30-45 kg m⁻³ during bathing). The oxygen level was either maintained throughout the bath or allowed to slowly decline to levels

approaching those of ambient (90-100%) by the end of the bath. After bathing, the tarpaulin liner was pulled away and the fish were released into the cage, which was then towed back to its original grow-out location. The freshwater quality parameters are shown in Table 7.2 (Powell et al. 2005).

7.3.2 Sample collection

Gill mucus samples were collected on five occasions (D0-PB, D49, D49-PB, D58 and D102) from March 2004 to July 2004 (Table 7.1). Day-zero samples were collected approximately after 2 h following the freshwater bath and it was considered day 0-post bath samples i.e. D0-PB. Similarly, 49 days after D0-PB sampling, D49 samples were collected. Immediately after D49 sample collection, the fish were exposed to freshwater and D49-PB samples were collected 2 h after the bath i.e. on the same day. D58 and D102 samples were collected on day 58 and day 102 of sampling respectively. At each time, 10 fish were euthanased with Aqui-S[®] anesthetic and gill mucus swabs were collected for bacteriology using sterile swabs (Medical Wire and Equipment Co. (Bath) Ltd., Wiltshire, England). Thus, at every sampling 10 swabs were collected, i.e. one swab per fish. Immediately after sampling for bacteriology, entire gills were collected for AGD assessment (results presented elsewhere by Powell et al. 2005; summarized in Table 7.1) and this included (1) scoring the gross AGD lesions, which are characterised by macroscopic focal or multifocal white mucoid patches on the gills using a method described by Clark and Nowak (1999); (2) estimating the number of amoeba on the gills using the method developed by Howard and Carson (1993); (3) recording the percentage of filaments with AGD lesions by viewing the histological sections of gills under a light

microscope using a method described by Adams and Nowak (2001). If a fish had gross AGD lesions and amoebae on the gills then the fish was considered AGD-positive regardless of the presence or absence of AGD lesions in the histological sections of the gill filaments. This study did not include any negative controls because it is not possible to find a fish in farm environment that are naïve to AGD.

7.3.3 Bacteriology

After collection, the swabs were placed at 0°C to 4°C and transported to the laboratory and were inoculated within 24 to 36 h of collection onto marine agar (refer to appendix 1 for media formulation), one swab per plate. The plates were incubated at 22°C for 24 to 96 h. Bacterial growth was examined on all plates and distinct colonies from each plate was subcultured to obtain pure colonies (refer to chapter 2, section 2.3 for details on bacteria isolation and morphological characterisation). The pure colonies were cryopreserved at -80°C for subsequent 16S rRNA analysis (refer to chapter 2, section 2.4 for details on cryopreservation procedure).

7.3.4 16S rRNA analysis

The cryopreserved samples were revived on their respective culture medium and were used for sequencing the 16S ribosomal RNA (rRNA). Please refer to chapter 2, sections 2.8, 2.9, 2.10 and 2.11 respectively for details on extraction of bacterial DNA, polymerase chain reaction (PCR), analysis of sequences and sequence identification. Sequences obtained in this study have been deposited under GenBank accession numbers DQ873735 to DQ873806.

7.4 Results

This study was conducted to obtain an approximate inference on the types of culturable bacterial colonies that were found on the fish gills in a given period of time and the frequency of its occurrence. A total of nine morphologically distinct bacterial phylotypes were identified during D0-PB sampling; similarly 10 isolates from D49, 19 each from D49-PB and D58 and 15 from D102 were identified on the gills of marine farmed Atlantic salmon gills during this study (Table 7.3). These bacteria are broadly classified into five groups based on their phyla/order (*Gammaproteobacteria*, *Cytophaga-Flavobacterium-Bacteroides* (CFB) phylum, Firmicutes, *Alphaproteobacteria* and *Actinobacteria*) and the numbers of isolates from each group are represented in the form of stacked graph for easy interpretation (Figure 7.1).

7.4.1 *Gammaproteobacteria* and *Alphaproteobacteria*

Gammaproteobacteria were commonly found on the gills during all sampling days (D0-PB to D102) (Figure 7.1). Majority ($\approx 48\%$ i.e. 14/29) of these *Gammaproteobacteria* grouped separately and belonged to the *Pseudoalteromonas* clade in the phylogenetic tree (Figure 7.2). The other *Gammaproteobacteria* that were sporadically isolated included *Cobetia* sp. (D0-PB, D49-PB, D102); *Halomonas ventosae* (D58); *Marinobacter* sp. (D102); *Psychrobacter* sp. (D102); *Pseudomonas* sp. (D102); *Enterobacter* sp. (D49-PB); *Vibrio splendidus* (D49); *Alteromonas* sp. (D0-PB) (Table 7.3). Among the *Alphaproteobacteria*, isolates closely related to *Alphaproteobacterium* sp. (D0-PB, D49-PB and D102) and *Paracoccus* sp. (D49-PB) were isolated (Figure 7.2).

7.4.2 *Cytophaga-Flavobacterium-Bacteroides* (CFB) phylum

CFB bacteria were identified on fish from D49, D49-PB, D58, and D102 samples (Figure 7.1). Two isolates from D49 and five from D49-PB fish were distantly related to *Mesonia algae*, a moderately halophilic marine bacterium. Fish from D49-PB also had an isolate closely related to *Salegentibacter mishustinae*. Further, six isolates from D58 samples branched in the psychrophilic, *Polaribacter* group while four of the D58 isolates and an isolate from D102 belonged to the recently described marine genus *Bizionia*. Two phylotypes from D102 and one from D58 sampling were located in the *Winogradskyella* group (Figure 7.2; Table 7.3).

7.4.3 Firmicutes and Actinobacteria

Among the Gram-positives, D49 and D49-PB had both Firmicutes and Actinobacteria on the gills, while D58 samples had only Firmicutes (Figure 7.1). Majority of the phylotypes from these sampling points (D49 = 2 isolates; D49-PB = 3 isolates and D58 = 3 isolates) grouped in the genus *Planococcus*. The other Firmicutes less frequently isolated were *Staphylococcus* spp. (D49-PB and D58), *Salinicoccus* sp. (D49-PB) and *Planomicrobium* (D58). Actinobacteria from D49 and D49-PB samples were closely related to the genera *Kocuria* and *Microbacterium* respectively (Table 7.3; Figure 7.2).

7.4.4 Comparison of pre (D49) and post (D49-PB) bathed samples

Both D49 and D49-PB samples were collected on the same day i.e. 49 days after D0-PB sampling; however, D49 samples were collected before freshwater

exposure and D49-PB samples were collected after freshwater exposure. Isolates closely linked to *Pseudoalteromonas*, *Mesonina algae* and *Planococcus* spp. were common to D49 and D49-PB samples. D49 samples also had a *Vibrio splendidus* and a *Kocuria* sp., while a phylotype closely linked to *Enterobacter amnigenus* and another showing 99% similarity to *Salegentibacter mishustinae* were detected only on D49-PB samples. Further, two isolates closely related to halotolerant *Cobetia* sp. and an isolate each from the genera *Salinicoccus*, *Staphylococcus* and *Microbacterium* were isolated only from D49-PB fishes.

7.4.5 Comparison of post bath samples (D0-PB and D49-PB)

D0-PB and D49-PB samples were collected on day 0 and day 49 respectively following a freshwater bath event. Both groups had *Pseudoalteromonas* sp., *Cobetia* sp. and *Alphaproteobacterium* sp. in common. *Alteromonas* sp. was only isolated from D0-PB samples; similarly an isolate closely linked to *Enterobacter* sp. was only present on D49-PB samples. D49-PB samples had bacteria belonging to CFB group such as *Mesonina* sp. and *Salegentibacter* sp.; however, none of the gill mucus samples obtained from D0-PB fish had CFB group bacteria. In addition, Gram-positives were absent in D0-PB samples, while D49-PB fish had three *Planococcus* spp., a *Staphylococcus* sp. and a *Salinicoccus* sp.. The main reason for the dissimilarity in the gill bacterial community in D0-PB and D49-PB samples might be the difference in freshwater temperature and salinity used during AGD treatment (Table 7.2), in addition to the variation in AGD severity and seawater temperature (Table 7.1).

Table 7.1 A summary table showing sampling details, freshwater treatment time, seawater temperature (temp.), AGD severity and amoebae number.

Sampling time	Sampling date	Treatment	Average fish weight (g)	Temp. (°C)	Mean % of filaments with AGD-lesions (\pm SE)	Gross AGD lesions	No. of amoeba on the entire gill surface
D0-PB	03/03/2004	Freshwater	717	16.8	10 (3.66)	1.3	3×10^5
D49	20/04/2004	-	906	14.8	0.62 (0.62)	1.7	40×10^5
D49-PB	20/04/2004	Freshwater	964	14.8	1.65 (0.90)	1.8	30×10^5
D58	04/05/2004	-	1132	14.0	1.60 (0.98)	0.6	50×10^5
D102	15/06/2004	-	1385	10.9	0 (0.00)	0.3	60×10^5

Table 7.2 Mean (\pm SE) water quality parameters

for freshwater bathing. ND = not determined

Water quality parameter	<u>Bathing event</u>	
	D0-PB 03/03/2004	D49-PB 20/04/2004
Hardness (mg l ⁻¹)	259.8	ND
Temperature (°C)	19.6 (0.00)	14.5 (0.01)
Salinity (g l ⁻¹)	1.24 (0.01)	8.25 (0.40)
DO (% air saturation)	163.1 (1.6)	116.4 (0.7)
DO (mg l ⁻¹)	14.9 (0.1)	11.4 (0.1)
pH	7.54 (0.00)	8.36 (0.02)
ORP (mV)	50.4 (0.02)	41.3 (0.1)

Table 7.3 Phylogenetic affiliations and frequency of isolates

Phylogenetic grouping	D0- PB	D49	D49- PB	D58	D102	Nearest phylogenetic relative *	% identical 16S rRNA †
<u>Gammaproteobacteria</u>							
<i>Cobetia</i>	1		2		1	<i>Cobetia</i> sp. SB J92 (AB167062)	98.5–98.8
<i>Halomonas</i>				1		<i>Halomonas ventosae</i> (AY268080)	99.4
<i>Marinobacter</i>					1	<i>Marinobacter</i> sp. DG979 (AY258112)	99.0
<i>Psychrobacter</i>					1	<i>Psychrobacter glacincola</i> (AJ430829)	96.2
					2	<i>Psychrobacter nivimaris</i> (AJ313425)	96.7, 98.1
<i>Pseudomonas</i>					1	<i>Pseudomonas mendocina</i> (AY870674)	99.5
					1	<i>Pseudomonas</i> sp. (AB021318)	99.1
<i>Enterobacter</i>			1			<i>Enterobacter amnigenus</i> (AB004749)	97.2
<i>Vibrio</i>		1				<i>Vibrio splendidus</i> (AJ874367)	99.1
<i>Alteromonas</i>	2					<i>Alteromonas</i> sp. No. 57 gene (AB180388)	97.8, 99.1
<i>Pseudoalteromonas</i>	3	3	1		2	<i>Pseudoalteromonas atlantica</i> (AB049728)	95.4–99.5
	1			2	1	<i>Pseudoalteromonas haloplanktis</i> (CR954246)	97.0–99.7
		1				<i>Pseudoalteromonas</i> sp. (DQ219365)	98.8
<u>CFB group</u>							
<i>Polaribacter</i>				6		<i>Polaribacter</i> sp. AC4 (DQ356493)	96.1–98.6
<i>Mesonina</i>		2	5			<i>Mesonina algae</i> KMM 3909 (AF536383)	96.3–99.5
<i>Bizionia</i>				4		<i>Bizionia paragorgiae</i> (AY651070)	97.4–99.0
					1	<i>Bizionia saleffrena</i> (AY694005)	96.1
<i>Winogradskyella</i>				1	1	<i>Winogradskyella thalassocola</i> (AY771720)	96.2–96.3
					1	<i>Winogradskyella</i> sp. BC1 (DQ356490)	95.5
<i>Salegentibacter</i>			1			<i>Salegentibacter mishustinae</i> (AY576653)	99.1
Unaffiliated lineages			1			<i>Salegentibacter</i> sp. 18III/A01/068i (AY576719)	93.7
<u>Alphaproteobacteria</u>							
<i>Alphaproteobacterium</i>	1		1		2	<i>Alpha proteobacterium</i> Med4c1:1 (AF493974)	98.0–99.6
<i>Paracoccus</i>			1			<i>Paracoccus</i> sp. HZ04 (AY690705)	99.1
Unaffiliated lineages	1					<i>Alpha proteobacterium</i> GMD29E5 (AY162072)	92.3
<u>Firmicutes</u>							
<i>Planococcus</i>		2	3	3		<i>Planococcus maritimus</i> TF-9 (AF500007)	95.6–98.8
<i>Salinicoccus</i>			1			<i>Salinicoccus</i> sp. (AJ717731)	97.8
<i>Staphylococcus</i>			1			<i>Staphylococcus pasteurii</i> U523B6 (DQ298130)	100.0
				1		<i>Staphylococcus equorum</i> (AY741060)	98.0
<i>Planomicrobium</i>				1		<i>Planomicrobium okeanokoites</i> (AY730709)	96.6
<u>Actinobacteria</u>							
<i>Kocuria</i>		1				<i>Kocuria</i> sp. S26-8 (DQ060377)	96.6
<i>Microbacterium</i>			1			<i>Microbacterium keratanolyticum</i> (Y14786)	96.8
Total # of distinct strains isolated	9	10	19	19	15		

Unaffiliated lineages means isolates which cannot be assigned to an established genus due to homology value of <95% with the reference sequence (Bosshard et al. 2003). * and † obtained from GenBank nucleotide database

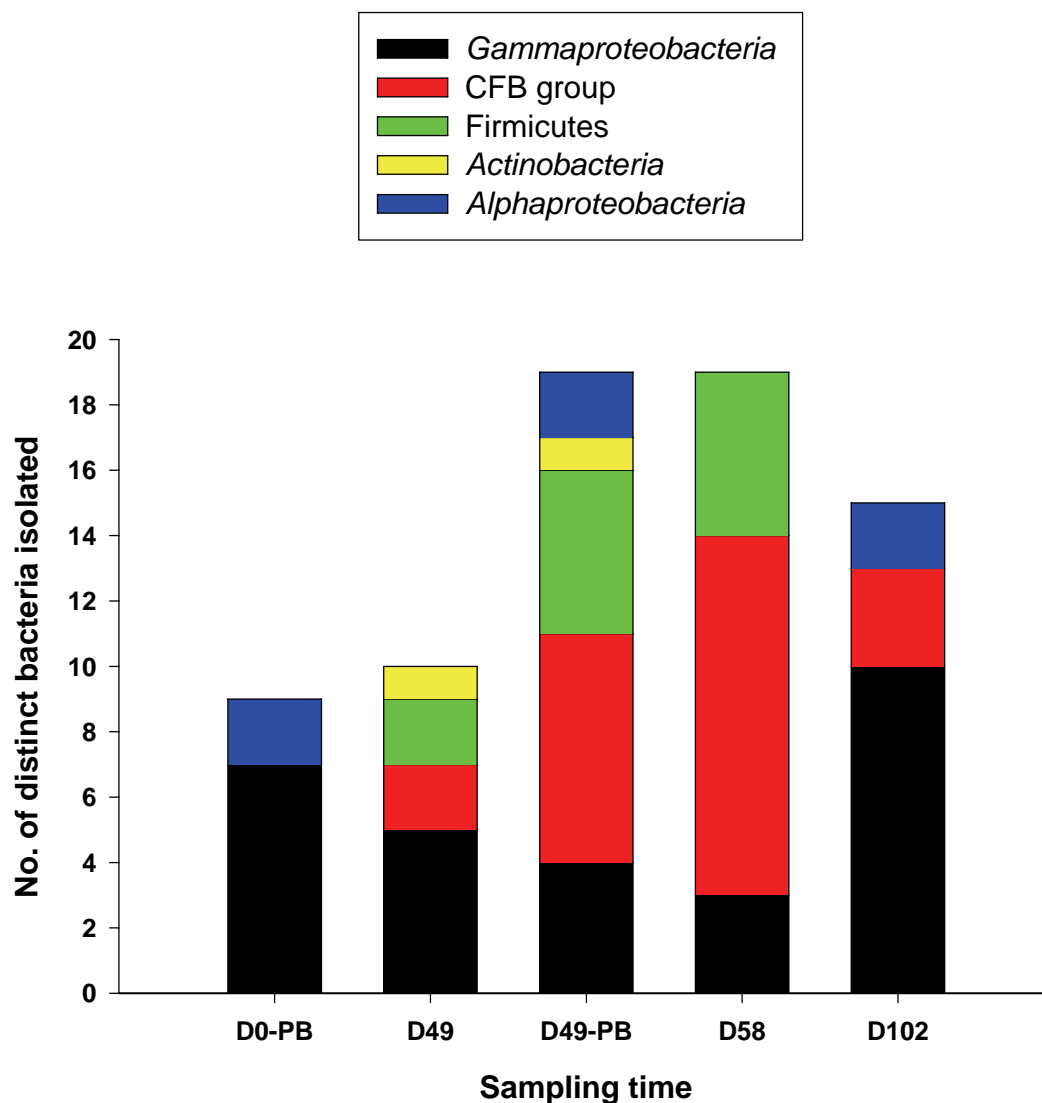
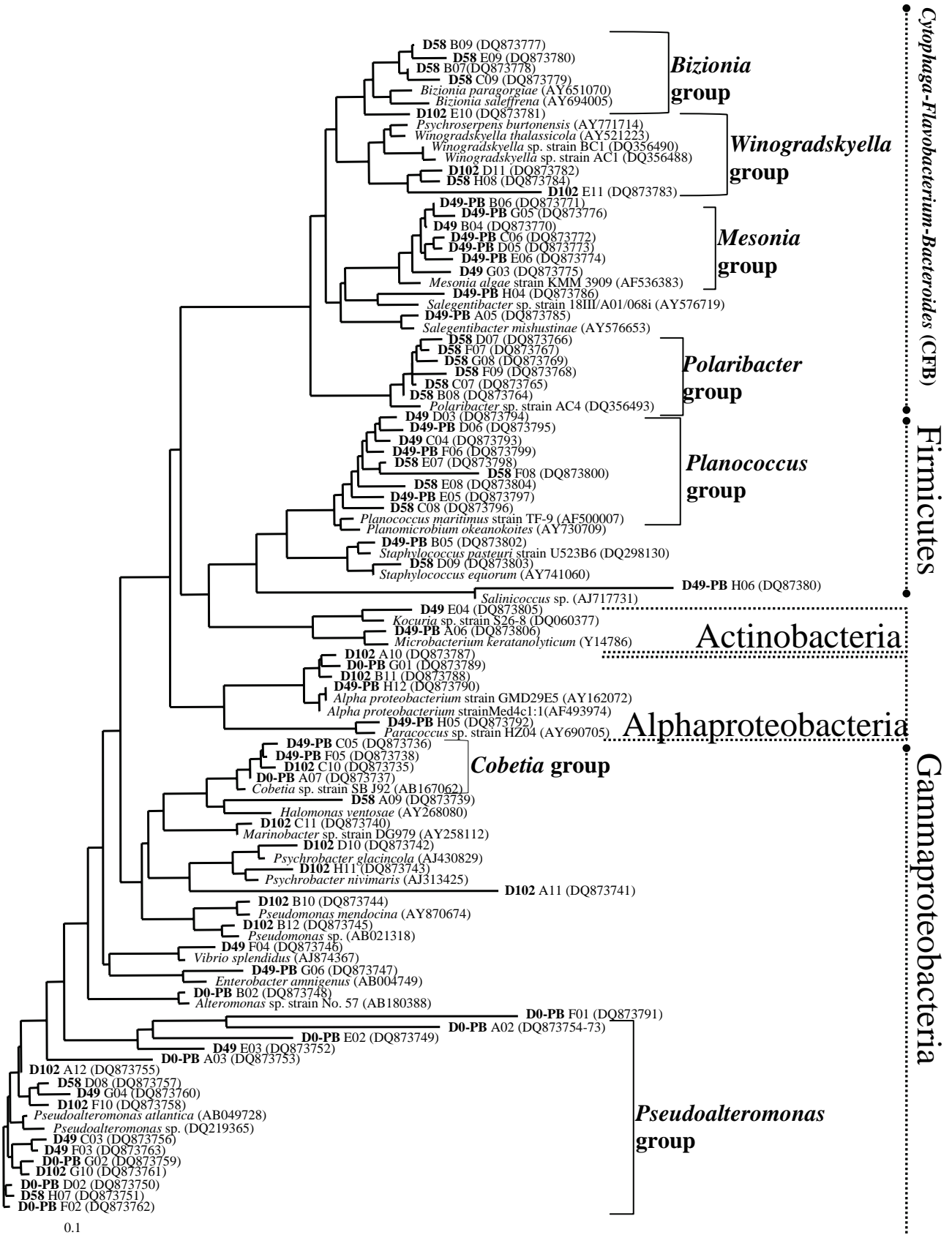


Figure 7.1 Composition of distinct bacterial species isolated from salmonid gills. Bars represent the abundances of different prokaryote groups (Firmicutes, *Actinobacteria*, *Cytophaga-Flavobacterium-Bacteroidetes* (CFB) group, *Alphaproteobacteria*, and *Gammaproteobacteria*) during various sampling times (D0-PB, D49, D49-PB, D58 and D102).

Figure 7.2 16S rRNA gene-based phylogenetic tree indicating the location of distinct bacterial isolates cultured from AGD-affected Atlantic salmon gills on five (D0-PB, D49, D49-PB, D58 and D102) different occasions over a period of 102 days. Scale bar indicates maximum likelihood evolutionary distance. Numbers in parenthesis are GenBank accession numbers. Figure 7.2 is shown in next page.



7.5 Discussion

A culture-based study was conducted to obtain a snapshot of the bacterial species present on Atlantic salmon gills during a typical occurrence of amoebic gill disease in a farm environment over a period of 102 days. The 16S rRNA analysis identified *Gammaproteobacteria* to be commonly found on the gills during all sampling days. Bacteria from other phyla/order were also isolated during this study and included CFB group bacteria, *Alphaproteobacteria*, Firmicutes, and *Actinobacteria*.

Winogradskyella species were not consistently isolated from AGD-affected fish gills during this study. Only D58 and D102 samples had *Winogradskyella* spp. on the gills, this genus was not isolated from other samples (D0-PB, D49 and D49-PB), suggesting that these bacteria may not be frequently present on AGD-affected fish gills at least at this farm. One of the possible reasons for not isolating these species during D0-PB and D49-PB sampling might be due to rapid lysis of *Winogradskyella* cells when exposed to low salinity water i.e. during freshwater bathing; because these species require Na⁺ for growth (Nedashkovskaya et al. 2005). Perhaps, *Winogradskyella* spp. may have the capacity to recolonise the gills from the surrounding seawater following exposure to low salinity as evident from D58 (i.e. 9 days after freshwater treatment) and D102 (i.e. 53 days post freshwater treatment) samples. However, *Winogradskyella* spp. were not isolated from D49 samples (i.e. 49 days post freshwater treatment); the main reason for this might be the difference in freshwater salinity used for bathing the fish preceding the sampling day i.e. during

D0-PB. The freshwater salinity was lower during D0-PB (1.24 g l^{-1}) compared to the salinity used during D49-PB (8.25 g l^{-1}) bathing. Hence, this difference in salinity of freshwater during D0-PB might have delayed the recolonisation of *Winogradskyella* spp. onto D49 fish gills. Perhaps, the number of amoebae on the gills could also influence the presence of *Winogradskyella* spp. on the gills because D58 and D102 had more number of *Neoparamoeba* spp. on the gills compared to D0-PB, D49 and D49-PB samples. In addition, there are several other factors that could have delayed/prevented the growth on *Winogradskyella* spp. on D49 samples; some of these factors can be noted from Tables 7.1 and 7.2. Nevertheless, further research is required to determine the correlation between these factors and the occurrence of *Winogradskyella* spp. on the gills.

Bacteria from the genus *Pseudoalteromonas* were predominantly isolated during this study. Members of the genus *Pseudoalteromonas* are widely distributed in the marine environment (Skovhus et al. 2004). Bacteria closely related to the genus *Pseudoalteromonas* were detected from the sediment samples obtained from the salmon sea cage sites in Tasmania (Bissett et al. 2006a). A culture-independent study detected two phylotypes closely related to a *Pseudoalteromonas* species (*P. antarctica*) only on the gills of AGD-affected Atlantic salmon (Bowman and Nowak 2004). Some *Pseudoalteromonas* species are reported to cause disease in fish and produce potent toxins, while others appear less virulent and may even be beneficial to their hosts (Holmström and Kjelleberg 1999). Furthermore, a study examining the antifouling activities of 10 different *Pseudoalteromonas* species reported that *P.*

tunicate and *P. ulvae* displayed the highest level of antifouling activity and prevented the settlement of invertebrate larvae, algal spores, bacteria and fungi on the marine surfaces (Holmström et al. 2002). Based on these results, it is evident that *Pseudoalteromonas* species could exhibit a diverse range of roles. Therefore, it might be worthwhile to verify the role of *Pseudoalteromonas* species in AGD.

Some of the bacteria isolated during this study have been identified to have pathogenic potential. For instance, a *Vibrio splendidus* isolate was obtained during D49 sampling. These species are reported to cause haemorrhages in turbot, *Scophthalmus maximus* (Angulo et al. 1994). Similarly, isolates closely related to *Alteromonas* spp. (D0-PB), *Planococcus* spp. (D49, D49-PB, D58), *Staphylococcus* spp. (D49-PB, D58) were cultured from AGD-affected fish gills during this study. Previous research reported that some *Alteromonas* species could cause necrosis and septicemia in oysters (Buller 2004). *Planococcus* spp. were presumed to cause mortalities in rainbow trout fry (Austin and Stobie 1992). *Staphylococcus epidermis* is pathogenic to Rea sea bream, *Chrysophrys major* and yellow tail, *Seriola quinqueradiata* (Kusuda and Sugiyama 1981) and *S. warneri* is pathogenic to rainbow trout (Gil et al. 2000).

Among CFB-group bacteria, *Mesonia algae*, a moderately halophilic marine bacterium was isolated from D49 and D49-PB samples; and 6/19 (32%) isolates from D58 samples were closely related to the genus *Polaribacter*. Previous studies on salmonid gill bacteria did not report the presence of *Mesonia* spp. (Trust 1975; Bowman and Nowak 2004; chapter 3). However, species closely related to *Polaribacter irgensii* were isolated from AGD-affected farm fish in our previous study (chapter 3). Additionally, bacteria from the genus *Polaribacter* were reported to be one of the predominant members among other CFB bacteria in the sediment samples obtained from the salmon sea cage sites in Tasmania (Bissett et al. 2006a). Likewise, the diversity of the CFB group was clearly reduced and concentrated within the *Polaribacter* group from the samples collected from Antarctic pack ice compared to Arctic pack ice isolates (Brinkmeyer et al. 2003). These results suggest that bacteria from the genus *Polaribacter* might be common in marine environment particularly in the southern ocean.

Alphaproteobacteria displaying a wide diversity were observed in marine sediment samples obtained from salmon farms located in Tasmania (Bissett et al. 2006a). Likewise, *Alphaproteobacteria* were the most numerous both in terms of the number of isolates cultured and were also the most abundant type of bacteria associated with the dinoflagellate *Gymnodinium catenatum* isolated from various locations including Tasmania (Green et al. 2004). However, *Alphaproteobacteria* were only sporadically isolated on salmonid gills during this study.

The majority of the Firmicutes from D49, D49-PB, and D58 sampling points grouped in the genus *Planococcus*. The other Firmicutes less frequently isolated included *Staphylococcus* sp. (D49-PB and D58), a *Planomicrobium* sp. (D58) and a *Salinicoccus* sp. (D49-PB). *Actinobacteria* closely related to the genera *Kocuria* (D49), and *Microbacterium* (D49-PB) were isolated. Similarly, the culture-independent study conducted by Bowman and Nowak (2004) identified a diverse range of Firmicutes and *Actinobacteria* including bacteria from the genera *Streptococcus*, *Staphylococcus*, *Kocuria*, *Arthrobacter*, *Frankia*, *Corynebacterium* on AGD-affected and unaffected fish gills. In addition, *Staphylococcus* spp. were frequently isolated from AGD-affected Atlantic salmon gills during the culture-dependent study identifying bacteria on salmonid gills (chapter 3).

In conclusion, during this study a diverse range of bacteria were isolated from Atlantic salmon gills. *Winogradskyella* spp., which is known to exacerbate AGD were not frequently isolated from AGD-affected fish gills. However, some of the bacteria (e.g. *Pseudoalteromonas* spp., *Vibrio* sp., *Staphylococcus* spp. and *Planococcus* spp.) isolated during this study are identified as potential fish pathogens. This might suggest that the onset of AGD in Atlantic salmon might favour the growth of any of these bacteria as secondary pathogens. However, it is not possible to determine which bacteria will colonise the gills of AGD affected fish in a given period of time because fish gills are in close contact with water and therefore the development of gill bacterial population is also dependent on various environmental factors affecting the aquatic ecosystem apart from the changes due to freshwater

bathing and AGD status of fish. Therefore, it appears that the trends/changes occurring within the bacterial community are complex and unpredictable. As a result, it could be stated that the bacterial community structure on the gills is determined by the environment and random succession events rather than proceeding in an easily predictable manner, which is same as the bacterial communities in marine sediments (Reice 1994; Bissett et al. 2006a).

Chapter 8

General Discussion

General Discussion

This thesis investigated the role of bacteria in amoebic gill disease (AGD). The main findings of this study are that AGD can occur at low levels of bacteria on the gills and that the disease could be exacerbated in the presence of *Winogradskyella* sp. strain AC1 (chapters 5 and 6). In addition, this study found that the bacteria belonging to *Cytophaga-Flavobacterium-Bacteroides* (CFB), Firmicutes and *Gammaproteobacteria* groups frequently colonise the gills of AGD-affected Atlantic salmon located in a farm (chapters 3 and 7).

This study demonstrated that *Neoparamoebae* are able to colonise disinfected gills i.e. gills containing low levels of bacteria due to potassium permanganate treatment (chapter 4, 5 and 6), signifying that the presence of large numbers of bacteria on the gills is not a prerequisite for *Neoparamoeba* spp. infection. This finding suggests that *Neoparamoeba* spp. is the primary causative agent of AGD. In addition, several laboratory based AGD trials (e.g. Munday et al. 2001; Adams and Nowak 2003, 2004) including the *in vivo* trials conducted during this study (chapters 4, 5 and 6) have confirmed that challenging fish exclusively with *Neoparamoeba* spp. could generate characteristic AGD lesions on the gills, similar to those lesions observed on the histological gill sections obtained from AGD-affected farm fish. In accordance with all the AGD research undertaken in Tasmania, a fish is diagnosed with AGD, if the histological section of the gills is characterized by a single or multifocal epithelial hyperplasia of the lamellae often containing round to ovate

interlamellar vesicles and *Neoparamoeba* spp. with a parasome. On the contrary, Bermingham and Mulcahy (2006) have suggested that not only *Neoparamoeba* spp. but also other organisms including other amoebae and protozoans are associated with AGD. However, the histological evidence provided by Bermingham and Mulcahy (2006) in their farm based study conducted in Ireland do not agree with the AGD pathology described in Tasmania, and the authors have not proved the identity or pathogenicity of the amoebae isolated in their study (Bermingham and Mulcahy 2006). Therefore, it appears that the disease described in Ireland is probably a mixed protozoan infection rather than AGD. In another case, an *in vitro* study examining the cell-pathogen interactions between a *Neoparamoeba pemaquidensis* strain and rainbow trout gill cell line suggested that the cytopathic effect of *Neoparamoeba* might be due to the extruded bacteria that the amoebae might have carried (Lee et al. 2006). On the other hand, these authors have not provided any histology or electron microscopic evidence to prove that the extruded bacteria caused the pathology; their proposition was exclusively based on the observation made through a phase contrast microscopy (Lee et al. 2006). In summary, the results presented in this thesis and other available evidence strongly suggest that *Neoparamoeba* spp. is the primary causative agent of AGD.

The current study also found that the fish experimentally infected with *Winogradskyella* sp. strain AC1 showed increased numbers of gill lesions following *Neoparamoeba* spp. infection (chapters 5 and 6), compared to those exposed to *Staphylococcus* sp. strain AC8 and *Neoparamoeba* spp. or infected with *Neoparamoeba* spp. alone (chapter 5). A previous study examining the interactions

between *Entamoeba histolytica* (an intestinal parasite and the causative agent of amoebiasis in humans) and bacteria has reported a similar outcome. Pre-incubation of *E. histolytica* with some Gram negative bacteria enhanced the virulence of amoebae and their ability to destroy monolayers of tissue-cultured cells (Bracha and Mirelman 1984). It was suggested that for the stimulation to be noted, *Entamoeba* trophozoites need to ingest bacteria and the ingested bacteria contribute a proteinaceous ingredient or enzymatic activity that rapidly activates a virulent response in the trophozoites (Bracha and Mirelman 1984; reviewed by Mirelman 1987). However, in case of *Neoparamoeba* and *Winogradskyella* species the interaction is not obvious because the ultrastructural studies using electron microscopy show that *Neoparamoeba* trophozoites fixed *in situ*, when attached to gill tissue do not contain bacteria (Dyková and Lom 2004). In addition, the histological examination of Atlantic salmon gills 4 days post exposure to *Winogradskyella* sp. strain AC1 showed that this bacterium is not detrimental to the fish (chapter 6). In other words, these findings suggest that the gill attached amoebae are not bacterivorous and *Winogradskyella* sp. may not be pathogenic to Atlantic salmon. Therefore, further research is required to determine the reasons for the increase in AGD severity in the presence of *Winogradskyella* sp.. Perhaps, *Winogradskyella* sp. has some physiological effect on Atlantic salmon, which makes the fish more vulnerable to *Neoparamoeba* sp. infection. Therefore, future research should also investigate the influence of *Winogradskyella* sp. on the physiological condition of Atlantic salmon.

During this study, a diverse range of bacteria were isolated from Atlantic salmon gills (chapters 3 and 7). Among these, the bacteria belonging to *Cytophaga-Flavobacterium-Bacteroides* (CFB), *Gammaproteobacteria* and Firmicutes groups were frequently isolated from AGD-positive farm fish (chapters 3 and 7). However, this study could not investigate the culturable bacteria on the gills of AGD-negative farm fish, because it is not possible to find a fish naïve to AGD in a farm environment. At all Tasmanian salmonid farms except Macquarie Harbour (see chapter 1, section 1.2), *Neoparamoeba* spp. have been consistently isolated from the nets, seacages and in nearby sediments (see Tan et al. 2002; Crosbie et al. 2005), therefore, it is not possible to find a fish which definitely had no exposure to *Neoparamoeba* spp.. On the other hand, it was possible to compare the culturable gill bacteria on AGD-negative and positive Atlantic salmon that were reared in separate seawater filled tanks located in a laboratory and the results showed that the bacteria commonly isolated from AGD-negative lab fish were *Gammaproteobacteria* and the culturable bacteria on AGD-positive fish belonged to Firmicutes and CFB group (chapter 3). Based on these findings, it appears that the occurrence of AGD might mainly favour the growth of CFB group bacteria on Atlantic salmon gills (Figure 8.1). The presence of *Gammaproteobacteria* on both AGD-negative lab fish (chapter 3) and AGD-positive farm fish (chapter 7) may indicate that these bacteria are part of the normal gill flora and are independent of AGD status of a fish. Nevertheless, whatever bacteria are present in the water column is likely to be present on fish (Shotts and Teska 1989). In this study, it was not possible to know if water-borne bacteria are just incidentally present or actually colonising the fish. Hence, there is always a possibility that the bacteria found in the water could also be on the

gills. Therefore, it is not possible to determine which bacteria commonly colonise the gills of AGD-affected fish. There is a strong association of CFB group bacteria with the marine water column, marine aggregates and with marine sediment communities around salmon farms (Ravenschlag et al. 2001; Bissett et al. 2006a) and perhaps, this is the reason for the presence of these bacteria on the fish gills. CFB group bacteria play a key role in the initial degradation of complex organic substrates, supplying hydrolysis and fermentation products for further mineralization (Rossello Mora et al. 1999; Kirchman 2002). In addition, CFB and *Gammaproteobacteria* have been identified to have a wide range of virulence and have been identified as opportunistic fish pathogens; but only a few studies have reported the bacteria from the phylum Firmicutes as disease causing agents in fish (see Austin and Austin 1993).

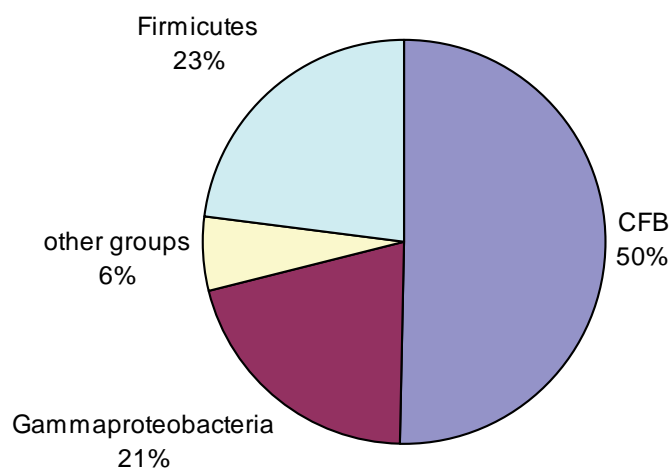


Figure 8.1 Composition of gill bacteria cultured during this study from AGD-affected Atlantic salmon (chapters 3 and 7).

The Firmicutes isolated from AGD-affected fish during this study mostly belonged to the genus *Planococcus* (chapter 7) and *Staphylococcus* (chapters 3 and 7). *Planococcus* spp. are presumed to cause mortalities in rainbow trout fry (Austin and Stobie 1992). *Staphylococcus warneri* is pathogenic to rainbow trout (Gil et al. 2000). The current study also isolated *Vibrio splendidus* (a *Gammaproteobacteria*), a pathogen reported to cause haemorrhages in turbot, *Scophthalmus maximus* (Angulo et al. 1994) from an AGD-positive fish (chapter 7). In addition, bacteria from the genus *Winogradskyella* (chapter 3) and *Psychroserpens* (Bowman and Nowak 2004) belonging to CFB group were detected in abundance only on the gills of AGD-affected Atlantic salmon. The genus *Psychroserpens* was established by Bowman et al. (1997) and contains *P. burtonensis* as the type species. *P. burtonensis* was first isolated from Burton Lake, Antarctica. There is no further information available on the association of *Psychroserpens* spp. with any organism. Meanwhile, the *in vivo* trials showed that the presence of a *Winogradskyella* sp. strain on salmonid gills during the development of AGD infection could result in a more severe AGD condition (chapters 5 and 6). Furthermore, the culture-independent study detected two *Tenacibaculum maritimum* (a CFB group bacterium) clones only on AGD-positive samples (Bowman and Nowak 2004). *T. maritimum* is the causative agent of flexibacteriosis in many marine fish species (Shotts and Teska 1989). In addition, bacteria from the genus *Pseudoalteromonas* (a *Gammaproteobacteria*) were more frequently isolated on AGD-positive fish gills (chapter 7). Some *Pseudoalteromonas* species are reported to cause disease in fish and produce potent toxins (Holmström and Kjelleberg 1999). Hence, considering the pathogenic potential of the above mentioned bacteria that were isolated from the gills of AGD-affected fish gills, it is

possible that the onset of AGD in Atlantic salmon might predispose the fish to colonisation by these bacteria as secondary opportunistic pathogens. Perhaps, similar to *Winogradskyella* sp., the presence of these bacteria on the gills during the process of AGD development may exacerbate the condition. However, further research is required to investigate the effect of other gill colonising bacteria isolated during this study in AGD.

Recently, a study examining the changes occurring in the sediment bacterial communities due to salmon farming in seacages in Tasmania reported that the sediment bacterial numbers including the CFB bacteria under salmon cages did not return to pre-stocking levels by the end of 3 months following period, but the bacterial numbers returned to the same as at the reference site (reference sites were locations with similar sediment characteristics, but without the organic loading/salmon-farming) levels over the following 12 months (Bissett et al. 2005; 2006b). CFB bacteria play a fundamental role in the initial degradation of organic matter, therefore their (CFB bacteria) numbers increase with increase in organic loading at the salmon farming sites (Bissett 2005). Furthermore, even though the ultrastructural studies conducted by Dyková and Lom (2004) suggested that gill attached *Neoparamoebae* may not be bacterivorous, the same study observed bacteria multiplying in the cytoplasm of trophozoites isolated from the nets and sediments in areas of salmon cages; thus, indicating that amoebae present on the nets and sediments might feed on bacteria. In addition, a significant association was found between *Neoparamoebae* densities and the number of bacteria during a spatial and temporal study assessing the distribution of amoeba in the water column around

salmon farm (Douglas-Helders et al. 2003). Further to this, the *in vitro* cultivation of *Neoparamoeba* strains has demonstrated that the amoebae could grow and multiply by feeding on live or heat-killed bacteria (Dyková et al. 2000, 2005; Morrison et al. 2005).

These findings have led to propose the following hypotheses:

- The primary diet of environmental strains of *Neoparamoeba* spp. could be bacteria.
- The increase in organic loading at salmon farming sites might increase bacterial numbers, which in turn may increase the amoebae numbers.
- Therefore, a prolonged fallowing period may reduce the bacterial numbers including the CFB group bacteria around the farm site. Consequently, this would lower the amoebae numbers because of lack of availability of food (bacteria).
- The presence of a CFB group bacterium (*Winogradskyella* sp. strain AC1) on AGD-affected fish gills was shown to intensify AGD severity (chapters 5 and 6) in laboratory condition. Therefore assuming that *Winogradskyella* sp. has similar effect on farmed fish, it is suggested that the absence or decrease in CFB bacteria numbers around the farm might decrease the possibility of occurrence of severe AGD outbreaks.
- Accordingly, the decrease in AGD severity could increase the time between fresh water bathing (a method to control AGD), which in turn will reduce the overall management costs spent towards controlling AGD. Corroborating this, Douglas-Helders et al. (2004) reported that rotation of stocked cages onto fallowed sites

significantly increased the time between freshwater baths compared to the bathing frequency requirements of the fish reared in stationary cages.

This study opted for culture-dependent methods (chapters 3 and 7) to identify salmonid gill bacteria instead of culture-independent approach. It is known that in the culture-dependent studies only a sub-population of bacteria is described. However, the culture-independent approach also has its own limitations; this method (culture-independent method) does not provide any information on the functions and interactions of the identified bacterium with other biota in the community (Carson et al. 2006). Therefore, when it comes to choosing an appropriate method, it all depends on the individual project and its aims. In the context of this project, using a culture method was most appropriate. For instance, in chapter 3, the cultivation method was adopted to obtain bacterial isolates from AGD-affected fish gills, so that these isolates could be used during *in vivo* trials to determine the role of bacteria in AGD. In chapter 7, the culture-dependent approach was applied in an attempt to verify whether *Winogradskyella* spp., previously shown to exacerbate AGD, frequently colonise the gills of sea-farmed Atlantic salmon. This was appropriate because results obtained from chapter 3 have shown that *Winogradskyella* spp. were culturable.

The isolated bacteria from this study were primarily grouped into respective clusters based on their biochemical characteristics and then from each group a typical isolate was selected for 16S ribosomal RNA (rRNA) analysis (see chapter 3). The disadvantage in using this method, is the assumption that all isolates in a cluster with

similar biochemical properties could have the same 16S rRNA identity as the representative isolate chosen for 16S rRNA analysis; however, there is an uncertainty whether every bacteria in a cluster is actually same as the representative isolate. This problem could be overcome by choosing every isolate from each cluster for 16S rRNA analysis. However, conducting both phenotypic and genotypic characterisation for each isolate could be a very time-consuming and expensive procedure. Several authors have reported that identification of bacteria using molecular techniques has provided reliable results (e.g. Wiik et al. 1995; Spanggaard et al. 2000; Bosshard et al. 2003, 2004; García-López et al. 2004). Therefore, instead of firstly classifying the bacteria phenotypically and then conducting the 16S rRNA analysis, it is proposed to use only genotypic identification for initial characterisation of bacteria. As a result, all the isolated bacteria were identified using 16S rRNA analysis alone in chapter 7. Nevertheless, for description of a novel species or for further studies it is necessary to use both phenotypic and genotypic procedures.

Overall, this study not only revealed the role of bacteria in AGD, it also led to few other important findings. This study has created a library for culturable bacteria present on AGD-affected salmonid gills (chapters 3 and 7). This research has also contributed more knowledge towards the recently established marine genus *Winogradskyella*. For example, we now know that short term exposure (4 days) of salmonids to *Winogradskyella* sp. strain AC1 at concentrations 10^8 or 10^{10} cells l^{-1} is not pathogenic; and presence of this bacterium on the Atlantic salmon gills could enhance the severity of AGD (chapter 5 and 6). In addition, this study has developed a protocol for disinfection of salmonid gills using potassium permanganate. This is

significant because the effect of this disinfection process has not been previously tested in conjunction with further disease challenge experiments (chapter 4). Furthermore, the results from this project indicate that further research could investigate the physiological effect of *Winogradskyella* sp. on Atlantic salmon, examine the relationship between *Winogradskyella* sp. and *Neoparamoeba* spp. and determine the possible reasons for the increase in AGD severity in the presence of this bacterium. Additionally, it would be interesting to examine the effects of pre-exposure of fish to much lower concentrations of *Winogradskyella* sp. (i.e. lower than 10^8 or 10^{10} cells l^{-1}) on AGD infection. Contamination of gills with very fine fish food particles may also affect the manifestation and severity of AGD. Furthermore, previous research has demonstrated that cultured gill-derived *N. pemaquidensis* are avirulent and therefore fails to elicit AGD in Atlantic salmon (Kent et al. 1988; Howard et al. 1993; Morrison et al. 2005); one of the reasons suggested for the down regulation of virulence in cultured amoebae was the absence of key nutritional factors required for virulence (Morrison et al. 2005). Therefore, it would be interesting to culture amoeba in the presence of *Winogradskyella* sp. and determine whether this has any effect on amoebae virulence. It is also intriguing to verify whether other gill colonising bacteria isolated during this study from AGD-affected Atlantic salmon gills has similar effect as *Winogradskyella* sp. has in exacerbating the AGD condition in Atlantic salmon.

References

References

Adams MB, Ellard K, Nowak BF (2004) Gross pathology and its relationship with histopathology of amoebic gill disease (AGD) in farmed Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases 27:151-161

Adams MB, Nowak BF (2001) Distribution and structure of lesions in the gills of Atlantic salmon, *Salmo salar* L., affected with amoebic gill disease. Journal of Fish Diseases 24:535-542

Adams MB, Nowak BF (2003) Amoebic gill disease: sequential pathology in cultured Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases 26:601-614

Adams MB, Nowak BF (2004) Experimental amoebic gill disease of Atlantic salmon, *Salmo salar* L.: further evidence for the primary pathogenic role of *Neoparamoeba* sp. (Page, 1987). Journal of Fish Diseases 27:105-113

Akhlaghi M, Munday BL, Rough K, Whittington RJ (1996) Immunological aspects of amoebic gill disease in salmonids. Diseases of Aquatic Organisms 25:23-31

Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25:3389-3402

- Amann R, Springer N, Schonhuber W, Ludwig W, Schmid EN, Muller KD, Michel R (1997) Obligate intracellular bacterial parasites of *Acanthamoebae* related to *Chlamydia* spp. Applied and Environmental Microbiology 63:115-121
- Angulo L, Lopez JE, Vicente JA, Saborido AM (1994) Haemorrhagic areas in the mouth of farmed turbot, *Scophthalmus maximus* (L.). Journal of Fish Diseases 17:163-169
- Austin B, Austin DA (1993) Bacterial fish pathogens : disease in farmed and wild fish, 2nd ed., Ellis Horwood, New York, pp. 364
- Austin B, Stobie M (1992) Recovery of *Micrococcus luteus* and presumptive *Planococcus* sp. from moribund fish during an outbreak of rainbow trout, *Oncorhynchus mykiss* (Walbaum), fry syndrome in England. Journal of Fish Diseases 15:203-206
- Bacon AS, Frazer DG, Dart JK, Matheson M, Ficker LA, Wright P (1993) A review of 72 consecutive cases of *Acanthamoeba* keratitis, 1984-1992 Eye 7:719-725
- Berk SG, Ting RS, Turner GW, Ashburn RJ (1998) Production of respirable vesicles containing live *Legionella pneumophila* cells by two *Acanthamoeba* spp. Applied and Environmental Microbiology 64:279-286

- Bermingham ML, Mulcahy MF (2006) Microfauna associated with amoebic gill disease in sea-farmed Atlantic salmon, *Salmo salar* L., smolts. *Journal of Fish Diseases* 29:455-465
- Bissett A (2005) Microbial ecology of fish farm sediments. PhD Thesis, University of Tasmania, Tasmania
- Bissett A, Bowman J, Burke C (2006a) Bacterial diversity in organically-enriched fish farm sediments. *FEMS Microbiology Ecology* 55:48-56
- Bissett A, Burke C, Cook PLM, Bowman JP (2006b) Bacterial Community Shifts in Organically Perturbed Sediments. *Environmental Microbiology* doi: 10.1111/j.1462-2920.2006.01110.x
- Bosshard PP, Abels S, Altwegg M, Bottger EC, Zbinden R (2004) Comparison of conventional and molecular methods for identification of aerobic catalase-negative gram-positive cocci in the clinical laboratory. *Journal of Clinical Microbiology* 42:2065-2073
- Bosshard PP, Abels S, Zbinden R, Bottger EC, Altwegg M (2003) Ribosomal DNA sequencing for identification of aerobic gram-positive rods in the clinical laboratory (an 18-month evaluation). *Journal of Clinical Microbiology* 41:4134-4140

- Bottone EJ, Madayag RM, Qureshi MN (1992) *Acanthamoeba* keratitis: Synergy between amebic and bacterial cocontaminants in contact lens care systems as a prelude to infection. *Journal of Clinical Microbiology* 30:2447-2450
- Bottone EJ, Perez AA, Gordon RE, Qureshi MN (1994) Differential binding capacity and internalisation of bacterial substrates as factors in growth rate of *Acanthamoeba* spp. *Journal of Medical Microbiology* 40:148-154
- Bowman JP, McCammon SA, Brown JL, Nichols PD, McMeekin TA (1997) *Psychroserpens burtonensis* gen. nov., sp. nov., and *Gelidibacter algens* gen. nov., sp. nov., psychrophilic bacteria isolated from Antarctic lacustrine and sea ice habitats. *International Journal of Systematic Bacteriology* 47:670-677
- Bowman JP, Nowak B (2004) Salmonid gill bacteria and their relationship to amoebic gill disease. *Journal of Fish Diseases* 27:483-492
- Bracha R, Mirelman D (1984) Virulence of *Entamoeba histolytica* trophozoites: effects of bacteria, microaerobic conditions, and metronidazole. *Journal of Experimental Medicine* 160:353-368
- Bridle AR, Carter CG, Morrison RN, Nowak BF (2005) The effect of beta-glucan administration on macrophage respiratory burst activity and Atlantic salmon, *Salmo salar* L., challenged with amoebic gill disease - evidence of inherent resistance. *Journal of Fish Diseases* 28:347-356

- Brieland J, McClain M, Heath L, Chrisp C, Huffnagle G, LeGendre M, Hurley M, Fantone J, Engleberg C (1996) Coinoculation with *Hartmannella vermiformis* enhances replicative *Legionella pneumophila* lung infection in a murine model of Legionnaires' disease. *Infection and Immunity* 64:2449-2456
- Brieland JK, Fantone JC, Remick DG, LeGendre M, McClain M, Engleberg NC (1997) The role of *Legionella pneumophila*-infected *Hartmannella vermiformis* as an infectious particle in a murine model of Legionnaires' disease. *Infection and Immunity* 65:5330-5333
- Brinkmeyer R, Knittel K, Jurgens J, Weyland H, Amann R, Helmke E (2003) Diversity and structure of bacterial communities in Arctic versus Antarctic pack ice. *Applied and Environmental Microbiology* 69:6610-6619
- Buller NB (2004) Bacteria from fish and other aquatic animals: A practical identification manual. CABI Publishing, Oxfordshire, UK, pp. 42
- Butler R, Nowak BF (2004) *In vitro* interactions between *Neoparamoeba* sp. and Atlantic salmon epithelial cells. *Journal of Fish Diseases* 27:343-349
- Carson J, Higgins MJ, Wilson TK, Gudkovs N, Bryant TN (2006) *Vibrios* of Aquatic Animals: Development of a National Standard Diagnostic Technology. In: Final report from Aquatic Animal Health Subprogram, FRDC Project No. 2001/628, pp. 158-161

- Cipriano RC, Ford LA, Teska JD, Hale LE (1992) Detection of *Aeromonas salmonicida* in the mucus of salmonid fishes. *Journal of Aquatic Animal Health* 4:114-118
- Cirillo JD, Cirillo SL, Yan L, Bermudez LE, Falkow S, Tompkins LS (1999) Intracellular growth in *Acanthamoeba castellanii* affects monocyte entry mechanisms and enhances virulence of *Legionella pneumophila*. *Infection and Immunity* 67:4427-4434
- Clark A, Nowak BF (1999) Field investigations of amoebic gill disease in Atlantic salmon, *Salmo salar* L., in Tasmania. *Journal of Fish Diseases* 22:433-443
- Clark G, Powell M, Nowak B (2003) Effects of commercial freshwater bathing on reinfection of Atlantic salmon, *Salmo salar*, with Amoebic Gill Disease. *Aquaculture* 219:135-142
- Crosbie PBB, Macleod C, Forbes S, Nowak BF (2005) Distribution of *Neoparamoeba* sp. in sediments around marine finfish farming sites in Tasmania. *Diseases of Aquatic Organisms* 67:61-66
- Darwish AM, Griffin BR, Straus DL, Mitchell AJ (2002) Histological and hematological evaluation of potassium permanganate exposure in Channel catfish. *Journal of Aquatic Animal Health* 14:134-144

- D'Aversa G, Stern GA, Driebe WT, Jr. (1995) Diagnosis and successful medical treatment of *Acanthamoeba* keratitis. Archives of Ophthalmology 113:1120-1123
- Douglas-Helders GM, Dawson DR, Carson J, Nowak BF (2002) Wild fish are not a significant reservoir for *Neoparamoeba pemaquidensis* (Page, 1987). Journal of Fish Diseases 25:569-574
- Douglas-Helders GM, Handlinger J, Carson J, Nowak BF (2003) Assessment over time of the infectivity of gill-derived and seawater dispersed *Neoparamoeba pemaquidensis* (Page, 1987). Bulletin of the European Association of Fish Pathologists 23:35-39
- Douglas-Helders GM, Saksida S, Nowak BF (2005b) Questionnaire-based risk assessment for amoebic gill disease (AGD) and evaluation of freshwater bathing efficacy of reared Atlantic salmon *Salmo salar*. Diseases of Aquatic Organisms 63:175-184
- Douglas-Helders GM, Weir IJ, O'Brien DP, Carson J, Nowak BF (2004) Effects of husbandry on prevalence of amoebic gill disease and performance of reared Atlantic salmon (*Salmo salar* L.). Aquaculture 241:21-30

- Douglas-Helders M, Nowak B, Butler R (2005a) The effect of environmental factors on the distribution of *Neoparamoeba pemaquidensis* in Tasmania. *Journal of Fish Diseases* 28:583-592
- Douglas-Helders M, Saksida S, Raverty S, Nowak BF (2001) Temperature as a risk factor for outbreaks of amoebic gill disease in farmed Atlantic salmon (*Salmo salar*). *Bulletin of the European Association of Fish Pathologists*. Weymouth 21:114-116
- Duncan TO (1974) A review of literature on the use of potassium permanganate (KMnO₄) in fisheries. Report FWS-LR-74-14, United States Fish and Wildlife Service, Washington, District of Columbia, USA.
- Dyková I, Figueras A, Novoa B (1995) Amoebic gill infection of turbot, *Scophthalmus maximus*. *Folia Parasitologica* 42:91-96
- Dyková I, Figueras A, Novoa B, Casal JF (1998) *Paramoeba* sp., an agent of amoebic gill disease of turbot *Scophthalmus maximus*. *Diseases of Aquatic Organisms* 33:137-141
- Dyková I, Figueras A, Peric Z (2000) *Neoparamoeba* Page, 1987: light and electron microscopic observations on six strains of different origin. *Diseases of Aquatic Organisms* 43:217-223

- Dyková I, Lom J (2004) Advances in the knowledge of amphizoic amoebae infecting fish. *Folia Parasitologica* 51:81-97
- Dyková I, Nowak BF, Crosbie PBB, Fiala I, Peckova H, Adams MB, Machackova B, Dvorakova H (2005) *Neoparamoeba branchiphila* n. sp., and related species of the genus *Neoparamoeba* Page, 1987: morphological and molecular characterization of selected strains. *Journal of Fish Diseases* 28:49-64
- Essig A, Heinemann M, Simnacher U, Marre R (1997) Infection of *Acanthamoeba castellanii* by *Chlamydia pneumoniae*. *Applied and Environmental Microbiology* 63:1396-1399
- Findlay VL, Munday BL (1998) Further studies on acquired resistance to amoebic gill disease (AGD) in Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases* 21:121-125
- Findlay VL, Zilberg D, Munday BL (2000) Evaluation of levamisole as a treatment for amoebic gill disease of Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases* 23:193-198
- Fisk DM, Powell MD, Nowak BF (2002) The effect of amoebic gill disease and hypoxia on survival and metabolic rate of Atlantic salmon (*Salmo salar*). *Bulletin of the European Association of Fish Pathologists* 22:190-194

- Ford LA, Barbash PA, Cipriano RC (1998) Control of furunculosis and enteric redmouth disease in sea-run Atlantic salmon broodstock in the Connecticut and Merrimack Rivers. *The Progressive Fish-Culturist* 60:88-94
- Francis-Floyd R, Klinger R (2003) Use of potassium permanganate to control external infections of ornamental fish. University of Florida, Institute of Food and Agricultural Sciences. <http://edis.ifas.ufl.edu/FA027>
- Fritsche TR, Horn M, Seyedirashti S, Gautom RK, Schleifer KH, Wagner M (1999) *In situ* detection of novel bacterial endosymbionts of *Acanthamoeba* spp. phylogenetically related to members of the order *Rickettsiales*. *Applied and Environmental Microbiology* 65:206-212
- Fritsche TR, Horn M, Wagner M, Herwig RP, Schleifer KH, Gautom RK (2000) Phylogenetic diversity among geographically dispersed *Chlamydiales* endosymbionts recovered from clinical and environmental isolates of *Acanthamoeba* spp. *Applied and Environmental Microbiology* 66:2613-2619
- Furuta K, Maruyama S (1981) Bacterial contamination on eggs during incubation and hatching, and of fluffs of newly-hatching, and of fluffs of newly-hatched chicks. *British Poultry Science* 22:247-254

- García-López I, Otero A, García-López M, Santos J (2004) Molecular and phenotypic characterization of nonmotile Gram-negative bacteria associated with spoilage of freshwater fish. *Journal of Applied Microbiology* 96:878-886
- Gil P, Vivas J, Gallardo CS, Rodriguez LA (2000) First isolation of *Staphylococcus warneri*, from diseased rainbow trout, *Oncorhynchus mykiss* (Walbaum), in Northwest Spain. *Journal of Fish Diseases* 23:295-298
- Gonzalez CJ, Lopez-Diaz TM, García-López ML, Prieto M, Otero A (1999) Bacterial microflora of wild Brown trout (*Salmo trutta*), Wild Pike (*Esox lucius*), and aquacultured rainbow trout (*Oncorhynchus mykiss*). *Journal of Food Protection* 62:1270-1277
- Green DH, Llewellyn LE, Negri AP, Blackburn SI, Bolch CJS (2004) Phylogenetic and functional diversity of the cultivable bacterial community associated with the paralytic shellfish poisoning dinoflagellate *Gymnodinium catenatum*. *Fems Microbiology Ecology* 47:345-357
- Greub G, Raoult D (2004) Microorganisms resistant to free-living amoebae. *Clinical Microbiology Reviews* 17:413-433
- Hadaś E, Derda M, Winiecka-Krusnell J, Sulek A (2004) *Acanthamoeba* spp. as vehicles of pathogenic bacteria. *Acta Parasitologica* 49:276-280

- Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41:95-98
- Harris JO, Powell MD, Attard M, Green TJ (2004) Efficacy of chloramine-T as a treatment for amoebic gill disease (AGD) in marine Atlantic salmon (*Salmo salar* L.). Aquaculture Research 35:1448-1456
- Hendrickson DA (1994) Reagents and Stains. In: Manual of Clinical Microbiology 4th edit., edited by Lennette, E. American society for Microbiology, Washington DC.
- Herwig N (1979) In: Handbook of drugs and chemicals used in the treatment of fish diseases. Charles C. Thomas Publisher, Spring Field, Illinois, pp.272
- Holmström C, Egan S, Franks A, McCloy S, Kjelleberg S (2002) Antifouling activities expressed by marine surface associated *Pseudoalteromonas* species. FEMS Microbiology Ecology 41:47-58
- Holmström C, Kjelleberg S (1999) Marine *Pseudoalteromonas* species are associated with higher organisms and produce biologically active extracellular agents. FEMS Microbiol Ecology 30:285-293
- Horn M, Harzenetter MD, Linner T, Schmid EN, Muller KD, Michel R, Wagner M (2001) Members of the *Cytophaga-Flavobacterium-Bacteroides* phylum as

- intracellular bacteria of *Acanthamoebae*: proposal of '*Candidatus Amoebophilus asiaticus*'. Environmental Microbiology 3:440-449
- Howard T, Carson J (1993) Development of a model infection from amoebic gill disease. In: Valentine P (ed) Proceedings of the Saltas Research and Development Review Seminar, Salmon Enterprises of Tasmania Pty. Ltd, Dover, Tasmania, pp.69-80
- Howard T, Carson J (1995) Amoebic gill disease laboratory research 1993/1994. In: Reports from the SALTAS 1993-1994 Research and development program, Salmon Enterprises of Tasmania Pty. Ltd, Dover, Tasmania, pp.71-87
- Inglis TJ, Rigby P, Robertson TA, Dutton NS, Henderson M, Chang BJ (2000) Interaction between *Burkholderia pseudomallei* and *Acanthamoeba* species results in coiling phagocytosis, endamebic bacterial survival, and escape. Infection and Immunity 68:1681-1686
- Jee LK, Plumb JA (1981) Effects of organic load on potassium permanganate as a treatment for *Flexibacter columnaris*. Trans. Am. Fish. Soc. 110:86-89
- Kahane S, Dvoskin B, Mathias M, Friedman MG (2001) Infection of *Acanthamoeba* polyphaga with *Simkania negevensis* and *S. negevensis* survival within amoebal cysts. Applied and Environmental Microbiology 67:4789-4795

- Kent ML, Sawyer TK, Hedrick RP (1988) *Paramoeba pemaquidensis* (*Sarcomastigophora: Paramoebidae*) infestation of the gills of Coho salmon *Oncorhynchus kisutch* reared in sea water. Diseases of Aquatic Organisms 5:163-169
- Kirchman DL (2002) The ecology of *Cytophaga-Flavobacteria* in aquatic environments. Fems Microbiology Ecology 39:91-100
- Kusuda R, Sugiyama A (1981) Studies on the characters of *Staphylococcus epidermidis* isolated from diseased fishes. Part 1. On the morphological, biological and biochemical properties. Fish Pathology 16:15-24.
- La Scola B, Raoult D (1996) Serological cross-reactions between *Bartonella quintana*, *Bartonella henselae*, and *Coxiella burnetii*. Journal of Clinical Microbiology 34:2270-2274
- Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackbrandt, E., Goodfellow, M. (ed.), Nucleic acid techniques in bacterial systematics. John Wiley and Sons, New York, pp. 135-142
- Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proceedings of the National Academy of Sciences USA 82:6955-6959

- Lau SCK, Tsoi MMY, Li X, Plakhotnikova I, Dobretsov S, Lau KWK, Wu M, Wong PK, Pawlik JR, Qian PY (2005) *Winogradskyella poriferorum* sp. nov., a novel member of the family *Flavobacteriaceae* isolated from a sponge in the Bahamas. *International Journal of Systematic and Evolutionary Microbiology* 55:1589-1592
- Lee LEJ, Van Es SJ, Walsh SK, Rainnie DJ, Donay N, Summerfield R, Cawthorn RJ (2006) High yield and rapid growth of *Neoparamoeba pemaquidensis* in co-culture with a rainbow trout gill-derived cell line RTgill-W1. *Journal of Fish Diseases* 29:467-480
- Leef MJ, Harris JO, Hill J, Powell MD (2005a) Cardiovascular responses of three salmonid species affected with amoebic gill disease (AGD). *Journal of Comparative Physiology* 175:523-532
- Leef MJ, Harris JO, Powell MD (2005b) Respiratory pathogenesis of amoebic gill disease (AGD) in experimentally infected Atlantic salmon *Salmo salar*. *Diseases of Aquatic Organisms* 66:205-213
- Lom J, Dyková I (1992) Protozoan parasites of fishes. In: *Developments in Aquaculture and Fisheries Science*, Vol. 26. Elsevier Science Publishers, Amsterdam. pp 77-86

- Masser MP, Jensen JW (1991) Calculating treatments for ponds and tanks. Southern Regional Aquaculture Centre (SRAC) Publication No. 410.
<http://wildlife.tamu.edu/1-8>
- Miles AA, Misra SS (1938) The estimation of the bacteriocidal power of blood. Journal of Hygiene-Cambridge 38:732-749
- Mirelman D (1987) Amoeba-bacterium relationship in amebiasis. Microbiological Reviews. 51:272-284
- Morrison RN, Crosbie PB, Nowak BF (2004) The induction of laboratory-based amoebic gill disease revisited. Journal of Fish Diseases 27:445-449
- Morrison RN, Crosbie PBB, Cook MT, Adams MB, Nowak BF (2005) Cultured gill-derived *Neoparamoeba pemaquidensis* fails to elicit amoebic gill disease (AGD) in Atlantic salmon *Salmo salar*. Diseases of Aquatic Organisms 66:135-144
- Munday BL (1986) Diseases of salmonids. In: Proceedings of the workshop on diseases of Australian fish and shellfish. Humphrey J.D. & Langdon J.S. (eds.) Department of Agriculture and Rural Affairs, Bennella, Victoria, pp.127-141
- Munday BL, Foster CK, Roubal FR, Lester RJG (1990) Paramoebic gill infection and associated pathology of Atlantic salmon, *Salmo salar*, and rainbow trout,

- Salmo gairdneri* (*Oncorhynchus mykiss*), in Tasmania. In: Pathology in marine science. Perkins FO and Cheng TC (eds.) Academic Press, San Diego, California, pp. 215-222
- Munday BL, Zilberg D, Findlay V (2001) Gill disease of marine fish caused by infection with *Neoparamoeba pemaquidensis*. Journal of Fish Diseases 24:497-507
- Murray CB, Evelyn TPT, Beacham TD, Barner LW, Ketcheson JE, Prosperi-Porta L (1992) Experimental induction of bacterial kidney disease in chinook salmon by immersion and cohabitation challenges. Diseases of Aquatic Organisms 12:91-96
- Nedashkovskaya OI, Kim SB, Han SK, Snauwaert C, Vancanneyt M, Swings J, Kim K-O, Lysenko AM, Rohde M, Frolova GM, Mikhailov VVa, Bae KS (2004) *Winogradskyella thalassicola* gen. nov., sp. nov., *Winogradskyella epiphytica* sp. nov. and *Winogradskyella eximia* sp. nov., marine bacteria of the family *Flavobacteriaceae*. International Journal of Systematic and Evolutionary Microbiology 55:49-55
- Noble AC, Herman RL, Noga EJ, Bullock GL (1997) Recurrent amoebic gill infestation in rainbow trout cultured in a semiclosed water recirculation system. Journal of Aquatic Animal Health 9:64-69

Nowak B, Munday BL (1992) In: Multi-Skilling in aquaculture a hands-on training workshop. Disease and Treatment. University of Tasmania.

Nowak BF (2001) Qualitative evaluation of risk factors for amoebic gill disease in cultured Atlantic salmon. In: Proceedings of the OIE International conference on risk analysis in aquatic animal health, Paris, France, 8-10 February, 2000. (Ed: Rodgers, C.J.). Office International des Epizooties (O.I.E.), pp.148-155

Nowak BF, Munday BL (1994) Histology of gills of Atlantic salmon during the first few months following transfer to seawater. Bulletin of the European Association of Fish Pathologists 14:77 - 81

Page RDM (1996) TREEVIEW: An application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences 12:357-358

Palzenberger M, Phola H (1992) Gill surface area of water breathing freshwater fish. Reviews in Fish Biology 2:187-216

Paniagua E, Parama A, Iglesias R, Sanmartin ML, Leiro J (2001) Effects of bacteria on the growth of an amoeba infecting the gills of turbot. Diseases of Aquatic Organisms 45:73-76

Parsons H, Nowak B, Fisk D, Powell M (2001) Effectiveness of commercial freshwater bathing as a treatment against amoebic gill disease in Atlantic salmon. Aquaculture 195:205-210

- Phillips HJ (1973) Dye exclusion test for cell viability. In: Kruse PF and Patterson MK (eds.) Tissue culture methods and applications. Academic Press, New York, pp. 406-408
- Powell MD, Attard M, Harris JO, Roberts SD, Leef MJ (2005) Why fish die - the treatment and pathophysiology of AGD. Aquafin CRC Project 3.4.1; FRDC project No. 2001/2005
- Powell MD, Clark GA (2004) Efficacy and toxicity of oxidative disinfectants for the removal of gill amoebae from the gills of amoebic gill disease affected Atlantic salmon (*Salmo salar* L.) in freshwater. Aquaculture Research 35:112-123
- Powell MD, Fisk D, Nowak BF (2000) Effects of graded hypoxia on Atlantic salmon infected with amoebic gill disease. Journal of Fish Biology 57:1047-1057
- Powell MD, Nowak BF, Adams MB (2002) Cardiac morphology in relation to amoebic gill disease history in Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases 25:209-216
- Powell MD, Parsons HJ, Nowak BF (2001) Physiological effects of freshwater bathing of Atlantic salmon (*Salmo salar*) as a treatment for amoebic gill disease. Aquaculture 199:259-266

- Ravenschlag K, Sahm K, Amann R (2001) Quantitative molecular analysis of the microbial community in marine Arctic sediments (Svalbard). *Applied and Environmental Microbiology* 67:387-395
- Reice SR (1994) Nonequilibrium determinants of biological community structure. *American Scientist* 82:424-435
- Rintamaeki P, Valtonen ET (1991) *Aeromonas salmonicida* in Finland: pathological problems associated with atypical and typical strains. *Journal of Fish Diseases* 14:323-331
- Roberts SD, Powell MD (2005) Oral L-cysteine ethyl ester (LCEE) reduces amoebic gill disease (AGD) in Atlantic salmon *Salmo salar*. *Diseases of Aquatic Organisms* 66:21-28
- Rossello-Mora R, Thamdrup B, Schafer H, Weller R, Amann R (1999) The response of the microbial community of marine sediments to organic carbon input under anaerobic conditions. *Systematic and Applied Microbiology* 22:237-248
- Roubal FR, Lester RJG, Foster CK (1989) Studies on cultured and gill-attached *Paramoeba* sp. (*Gymnamoebae*: *Paramoebidae*) and the cytopathology of paramoebic gill disease in Atlantic salmon, *Salmo salar* L., from Tasmania. *Journal of Fish Diseases* 12:481-492

- Sawyer ES (1978) Vibriosis in Maine and New Hampshire salmonids. *Marine Fisheries Review* 40:9-10
- Shah KL, Tyagy BC (1986) An eye disease in silver carp, *Hypophthalmichthys molitrix*, held in tropical ponds, associated with the bacterium *Staphylococcus aureus*. *Aquaculture* 55:1-4.
- Shaw BL, Battle HI (1957) The gross and microscopic anatomy of the digestive tract of the oyster *Crassostrea virginica*. *Canadian Journal of Zoology* 35:324-347
- Shotts EB, Teska JD (1989) Bacterial pathogens of aquatic vertebrates. In: Austin, B. and Austin, D.A. (eds) *Methods for the Microbiological Examination of Fish and Shell fish*. Ellis Horwood, Chichester, UK, pp.164-186
- Skovhus TL, Ramsing NB, Holmstrom C, Kjelleberg S, Dahlløf I (2004) Real-time quantitative PCR for assessment of abundance of *Pseudoalteromonas* species in marine samples. *Applied and Environmental Microbiology* 70:2373-2382
- Song YL, Fryer JL, Rohovec JS (1988) Comparison of six media for the cultivation of *Flexibacter columnaris*. *Fish Pathology* 23:91-94
- Soriano JM, Rico H, Manes J (2000) Assessment of the microbiological quality and wash treatments of lettuce served in University restaurants. *International Journal of Food Microbiology* 58:123-128

- Spanggaard B, Huber I, Nielsen J, Nielsen T, Appel KF, Gram L (2000) The microflora of rainbow trout intestine: a comparison of traditional and molecular identification. *Aquaculture* 182:1-15
- Speare DJ, Arsenault G, MacNair N, Powell MD (1997) Branchial lesions associated with intermittent formalin bath treatment of Atlantic salmon, *Salmo salar* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* 20:27-33
- Steinert M, Birkness K, White E, Fields B, Quinn F (1998) *Mycobacterium avium* bacilli grow saprozoically in coculture with *Acanthamoeba polyphaga* and survive within cyst walls. *Applied and Environmental Microbiology* 64:2256-2261
- Tan CKF, Nowak BF, Hodson SL (2002) Biofouling as a reservoir of *Neoparamoeba pemaquidensis* (Page, 1970), the causative agent of amoebic gill disease in Atlantic salmon. *Aquaculture* 210:49-58
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 24:4876-4882

- Trust TJ (1975) Bacteria associated with the gills of salmonid fishes in freshwater. Journal of applied bacteriology. Oxford Journal of Applied Bacteriology 38:225-233
- Tucker CS (1984) Potassium permanganate demand of pond waters. The Progressive Fish-Culturist 46:24-28
- Tucker CS, Boyd CE (1977) Relationships between potassium permanganate treatment and water quality. Transactions of the American Fisheries Society 106:481-488
- Valheim M, Haestein T, Myhr E, Speilberg L, Ferguson HW (2000) Varracalbmi: a new bacterial panophthalmitis in farmed Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases 23:61-70
- Ward ME, Watt PJ (1971) The preservation of gonococci in liquid nitrogen. Journal of clinical pathology 24:122-123
- Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 173:697-703
- Wiik R, Stackebrandt E, Valle O, Daae FL, Roedseth OM, Andersen K (1995) Classification of fish-pathogenic *Vibrios* based on comparative 16S rRNA analysis. International Journal of Systematic Bacteriology 45:421-428

Wishart D (1999) Clustan graphics primer: A guide to cluster analysis. Clustan Ltd, Edinburgh, Scotland, pp. 59

Wong FYK, Carson J, Elliott NG (2004) 18S ribosomal DNA-based PCR identification of *Neoparamoeba pemaquidensis*, the agent of amoebic gill disease in sea-farmed salmonids. Diseases of Aquatic Organisms 60:65-76

Zilberg D, Gross A, Munday BL (2001) Production of salmonid amoebic gill disease by exposure to *Paramoeba* sp. harvested from the gills of infected fish. Journal of Fish Diseases 24:79-82

Zilberg D, Munday BL (2000) Pathology of experimental amoebic gill disease in Atlantic salmon, *Salmo salar* L., and the effect of premaintenance of fish in sea water on the infection. Journal of Fish Diseases 23:401-407

Zilberg D, Nowak B, Carson J, Wagner T (1999) Simple gill smear staining for diagnosis of amoebic gill disease. Bulletin of the European Association of Fish Pathologists 19:186-189

Appendices

Appendix 1

Formulae of bacterial culture media (chapter 2)

Marine agar

Bacteriological peptone: 5 g

Yeast Extract: 2 g

Ferric pyrophosphate: 10 mg

Natural sea water (salinity 35‰): 1000 ml

pH 7.3-7.5

Autoclave 121°C for 15 min

Shieh's medium

(Song YL, Fryer JL, Rohovec JS (1988) Comparison of six media for the cultivation of *Flexibacter columnaris*. Fish Pathology 23:91-94)

Peptone (Oxoid L37): 5 g

Sodium acetate: 0.01 g

Sodium pyruvate: 0.1 g

Citric acid: 0.01 g

Yeast extract: 0.5 g

Agar: 12.0 g

Distilled water: 100 ml

Natural sea water (salinity 35‰): 900 ml

pH 7.5-7.8

Autoclave at 121°C for 15 min

Sheep blood agar with 2% NaCl

Blood agar base No. 2 (Oxoid, CM0271): 40 g

NaCl: 15 g

Distilled water: 1000 ml

pH: 7.4 ± 0.2

Autoclave at 121°C for 15 min and cool to 50°C; aseptically add 70 ml of defibrinated sheep's blood. Mix gently and pour as plates.

Appendix 2

Biochemical profiles of gill bacteria isolated from AGD-affected and unaffected salmonid gills (chapter 3)

Appendix 2 Biochemical profiles of the bacterial isolates obtained from group A (AGD affected fish from farm), group B (AGD affected laboratory fish) and group C (unaffected fish) fish. The isolates from group A are denoted as AC1 to AC10; group B as BC1 to BC4 and group C as CC1 to CC14. The cluster exemplars that were selected for 16S rRNA analysis are highlighted in grey.

[illegible]

[illegible]

1, Alkaline phosphate; 2, Esterase (C4); 3, Esterase Lipase (C8); 4, Lipase; 5, c Leucine arylamidase; 6, Valine arylamidase; 7, Cystine arylamidase; 8, Trypsin; 9, α -chymotrypsin; 10, Acid phosphatase; 11, Naphthol-AS BL Phosphohydrolase; 12, β -galatosisidase; 13, β -glucuronidase; 14, α -glucosidase; 15, N-acetyl- β -glucosaminidase; 16, α -mannosidase; 17, α -fucosidase; 18, Glycerol; 19, Erythritol; 20, L Arabinose; 21, L Arabinose; 22, Ribose; 23, D Xylose; 24, L Xylose; 25, Adonitol; 26, Galactose; 27, Glucose; 28, Fructose; 29, Mannose; 30, Sorbose; 31, Rhamnose; 32, Dulcitol; 33, Inositol; 34, Mannitol; 35, Sorbitol; 36, α -Methyl D Mannoside; 37, α -Methyl D Glucoside; 38, Arbutin; 39, Esculin; 40, Cellobiose; 41, Maltose; 42, Lactose; 43, Melibiose; 44, Sucrose; 45, Trehalose; 46, Melezitose; 47, Raffinose; 48, Starch; 49, Glycogen; 50, Xylitol; 51, Gentiobiose; 52, D-Turanose; 53, D-Lyxose; 54, D-Tagatose; 55, D-Fucose; 56, L-Fucose; 57, D- Arabitol; 58, L-Arabitol; 59, Gluconate; 60, 2-Keto Gluconate; 61, 5-Keto Gluconate; All isolates showed negative reaction to β -galatosisidase; α -mannosidase; Fructose; Maltose; Lactose; Trehalose; Melezitose; D-Turanose.