Investigations into the pathogenesis of aquatic *Streptococcus agalactiae* and *Streptococcus iniae* in Nile tilapia (*Oreochromis niloticus*)

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Abstract

The bacterial pathogens *Streptococcus agalactiae* and *S. iniae* have the capacity to infect a wide range of fish species throughout the world, with Nile tilapia (*Oreochromis niloticus*) being particularly susceptible. Global tilapia aquaculture production was estimated to be 3.5 million tonnes in 2008, and has a significant contribution in the global farmed fish market. Due to their ability to adapt to a wide range of culture systems the commercialisation of tilapia production has occurred in more than 100 countries. However, countries such as China have suffered from severe and extensive outbreaks of streptococcosis in cultured tilapia continuously for many years. Such large-scale outbreaks in China have resulted in a loss of approximately US$0.4 billion in 2011.

Fish are permanently exposed to a plethora of pathogens and natural disease outbreaks are complex host-pathogen interactions that seldom involve single pathogen infections. As a consequence, simultaneous infections, alternatively called concurrent or co-infections, are starting to receive interest from aquatic disease researchers.

Streptococcus agalactiae and *S. iniae* infections can both occur in the same geographic area and both *S. agalactiae* and *S. iniae* have been found to be present on the same farm in a single disease outbreak. It has been found that a disease outbreak caused by one these pathogens can be followed by another outbreak from the other. These two pathogens have serious effects on the tilapia aquaculture industry yet there is no information regarding *S. agalactiae* and *S. iniae* co-infections. Such information would be valuable for understanding epidemiology and the development of improved treatment and control of aquatic streptococcosis infections. The overall aim of this study was to investigate the pathogenesis of *S. agalactiae* and *S. iniae* in Nile tilapia.

One important aspect of investigating simultaneous infections was to examine if there was any competition or synergy between *S. agalactiae* and *S. iniae in vitro* or *in vivo.* It was found that competition between *S. agalactiae* and *S. iniae in vitro* was inconsistent between different experimental systems. Results indicated that there was either no interaction between bacterial species or they coexisted during *in vitro* competition assays. Whereas, an *in vivo* model utilising wax moth larvae (*Galleria mellonella*) suggested that during a simultaneous infection with *S. agalactiae* and *S. iniae* the total levels of larval mortality were lower than expected indicating that the pathogens may have interacted with one another in a competitive manner.

Investigations were also conducted to identify the expression of virulence factors *in vitro* for *S. agalactiae* and *S. iniae.* Comparisons were then made to ascertain any inter- and intra-species variation. Results demonstrated that both *S. agalactiae* and *S. iniae* strains possessed a capsule but varied in their haemolytic activity, blood survival and resistance to complement-mediated killing. These variations suggested that the two bacterial species differed in their mechanisms of pathogenicity where aquatic *S. agalactiae* strains may initially have a more systemic spread of infection and aquatic *S. iniae* strains may utilise a more localised spread of infection within the host. This hypothesis was tested through the development of a robust and reliable challenge model for *S. agalactiae* and *S. iniae* in Nile tilapia. Through this work it was apparent that fish infected with *S. iniae* experienced an acute infection with morbidity/mortality occurring 1 – 3 days after exposure. Whereas, the *S. agalactiae* challenged fish showed a more chronic infection with morbidity/mortality occurring from 1 – 6 days after exposure. Findings clearly demonstrated a more systemic spread of infection during a *S. agalactiae* challenge with high bacterial loads in all the organs examined. *Streptococcus iniae* was observed in fewer organs of infected fish and bacterial numbers were substantially lower.

Concurrent infections are complex in natural conditions and in experimental studies. As a result a substantial amount of research will be required to fully understand the nature of co-infection with these two streptococci. This study has provided a solid foundation upon which to base future work.

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Abbreviations and symbols

°	Degrees
°C	Degrees Celsius
%	Percentage
±	Plus or minus
<	Is less than
>	Is more than
≈	Approximately
µg	Microgram
µl	Microliter
µM	Micromolar
µm	Micrometre
α	Alpha
α-GAL	α galactosidase
β	Beta
β-GAL	β galactosidase
β-GUR	β glucuronidase
ADH	Arginine dihydrolase
AFLP	Amplified fragment length polymorphism
AMD	Amygdalin
AMP	Antimicrobial peptide
ANOVA	Analysis of variance
ARA	Arabinose
ATCC	American Type Culture Collection
BHIA	Brain Heart Infusion Agar
bp	Base pair
CAMP	Christie Atkins Munch Peterson
CCR	Carbon catabolite repression
cfu	Colony forming unit
cm	Centimetre
CNS	Central nervous system
Cpn60	Chaperonin 60 gene
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
E	Erythrocytes
ECP	Extracellular products
EDTA	Ethylenediaminete-traacetic acid
EGC	Eosinophilic granular cells
EPS	Exopolysaccharide/ Extracellular polysaccharide
ESC	Aesculin
F	Forward
FbsA	Fibrinogen-binding protein A
FCR	Feed conversion ratio
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenases
GAS	Group A streptococci
GBS	Group B streptococci
GFP	Green fluorescent protein
GLM	General linear model
GLYG	Glycogen
h	Hours
H&E	Haematoxylin and eosin
HCl	Hydrogen chloride
HIP	Hippurate
IFAT	Indirect fluorescent antibody technique
Ig	Immunoglobulin
IL-8	Interleukin-8 protease
INU	Inulin
i.p.	Intraperitoneal
i.m.	Intramuscular
Kb	Kilobase
kDa	Kilodalton
l	Litre
L	Ladder/ Lymphocytes
LAC	Lactose
LAMP	Loop-mediated isothermal amplification
LAP	Leucine arylamidase
lctO	Lactate oxidase-encoding gene
Lmb	Laminin-binding protein
LTA	Lipotechoic acid
M	Molar
mA	Milliampere
MAN	Mannitol
Met	Methionine
mg	Milligram
$MgCl_2$	Magnesium chloride
ml	Millilitre
mm	Millimetre

mM	Millimolar
MR-VP	Methyl-red and Voges-Proskauer
MW	Molecular weight
N	Neutrophils
n/a	Not available/Not applicable
NaCl	Sodium chloride
NCIMB	National Collection of Industrial, Food and Marine Bacteria
ng	Nanogram
nM	Nanomolar
nm	Nanometer
OD	Optical density
OF	Oxidation/fermentation
PAL	Alkaline phosphatase
PBS	Phosphate buffered saline
PCR	Polymerised chain reaction
ppm	parts per million
PYRA	Pyrrolidonylarylamidase
qPCR	Quantitative polymerised chain reaction
R	Reverse
RAF	Raffinose
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RFP	Red fluorescent protein
RIB	Ribose
rRNA	Ribosomal ribonucleic acid
s	Slow reaction
SBA	Sheeps blood agar
S.D	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
S.E.M.	Standard error of the Mean
SEM	Scanning Electron Microscope
Sia-CPS	Sialic acid capsular polysaccharide
SLS	Streptolysin S
SodA	Superoxidase dismutase
SOR	Sorbitol
sp./spp.	Species
STE	Sodium Chloride-Tris-EDTA
TAE	Tris-acetate-EDTA
TCS	Two-component system
TE	Tris-EDTA
TEM	Transverse electron microscope
TRE	Trehalose
TRIS	tris (hydroxymethyl) aminomethane
TSA	Tryptone soya agar
TSB	Tryptone soya broth
U	Units
UK	United Kingdom
US/USA	United States of America
UV	Ultraviolet
v	Variable
V	Volts
VIE	Visible implant elastomer
VP	Voges-Proskauer
v/v	Volume/Volume
v/w	Volume/Weight
W	Watts
w/v	Weight/Volume

Chapter 1

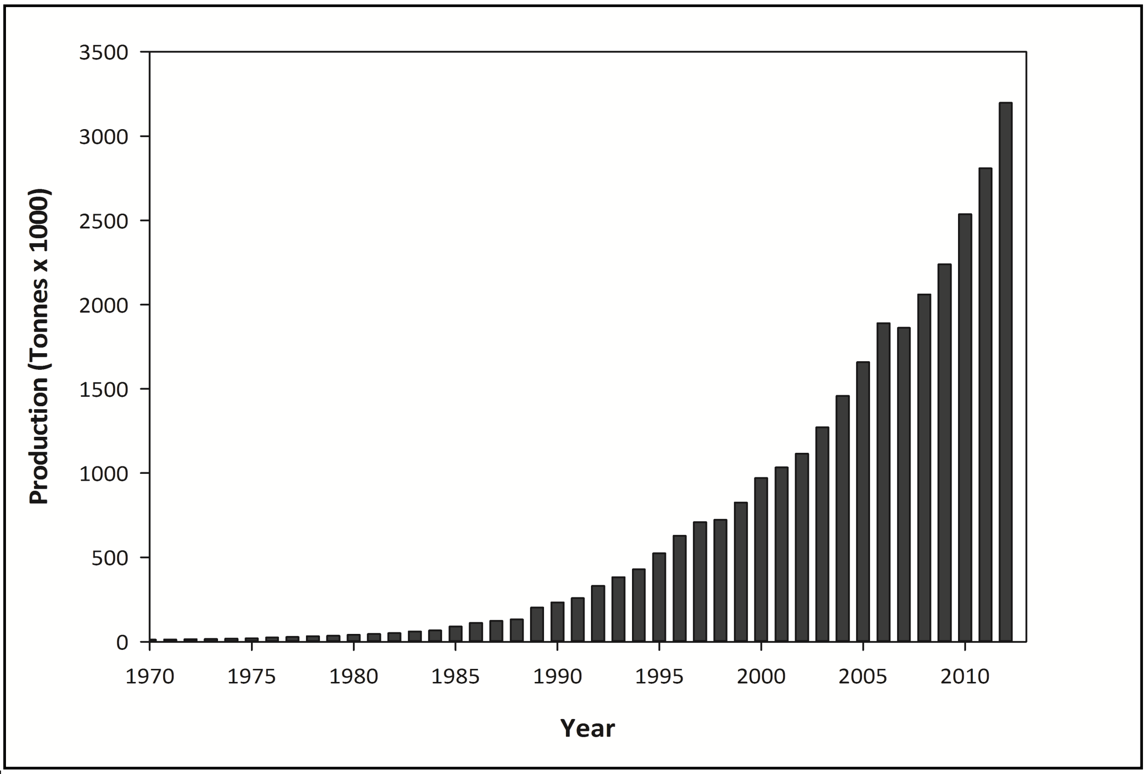
General introduction

1.1 Tilapia aquaculture

Since 1970 aquaculture has become the fastest growing food-producing sector in the world (Bondad-Reantaso *et al*., 2005), growing annually at an average of 6.6% (FAO, 2010). Global aquaculture (excluding aquatic plants) amounted to 52.5 million tonnes in 2008 with a value of US$98.4 billion (FAO, 2010). The epicentre of world aquaculture is in the Asia-Pacific region, generating 89% of total production and subsequently providing 79% of its value (FAO, 2010). A key aspect of the success of aquaculture in these countries has been the utilisation of introduced species.

Nile tilapia (*Oreochromis niloticus*) is native to the African continent but due to its favourable biological characteristics has thrived in various culture conditions around the world (Lazard, 1997). Distribution of Nile tilapia occurred from the 1960s but initial interest in this species was suppressed as uncontrolled breeding and inadequate husbandry techniques led to a low-percentage of marketable-sized fish (FAO, 2012). However, rapid expansion of this industry has occurred since the mid-1980s through the development and application of techniques such as male monosex populations (FAO, 2012). Due to the resulting uniformity of fish growth, allowing populations to reach marketable sizes, an unprecedented development of the industry occurred. This has been increased further by the aquaculture potential of this species: its hardiness, diverse feeding habits, high reproductive capability, success in various production systems, firm flesh texture and neutral flavour (Edwards *et al*., 2000; Lazard, 1997; Young and Muir, 2000). These product attributes have resulted in a widespread consumer appeal and thriving international trade (Figure 1.1). The farmed global tilapia production was estimated to be 3.5 million tonnes in 2008, of which, three quarters of this production was from Nile tilapia (Josupeit, 2010).

Due to their ability to adapt to a wide range of culture systems the commercialisation of tilapia production has occurred in more than 100 countries (FAO, 2012). Tilapia can be raised in an array of different locations, which have previously been categorised into three major resource zones: inland rural areas, periurban areas in wastewater-fed ponds, and in coastal areas in brackish water ponds (Edwards *et al.*, 2000). Currently, production of Nile tilapia occurs in a range of systems such as earthen ponds, floating cages, tanks and raceways and recirculation systems (FAO, 2012). The culture system selected is often dependent upon production intensity, culture sites, infrastructure, environmental conditions, socio-economic factors, access to technology and marketing potential (Gupta and Acosta, 2004).



[1]**Figure 1.1** Global aquaculture production of Nile tilapia. Data obtained from FAO (2012).

[1] Countries that report production values to FAO under the general statistical categories 'tilapias nei' (which may include other tilapia species) is not included in these total production values.

1.2 Aquatic diseases

Through increased globalisation of trade and market, there is an escalating demand for aquatic products. Consequently, farming sectors must undergo intensification and commercialisation to support global demand (Bondad-Reantaso *et al*., 2005). However, any intensification of livestock production comes with increased risk of disease; hence one key requirement for the culture of various aquatic species is health management in order to reduce the risk of major disease problems.

There are countless definitions of the precise meaning of disease however, Austin and Austin (2007) concluded that 'disease is a complex phenomenon leading to some form of measureable damage to the host'. The appearance and development of a fish disease is considered to be the outcome of an interaction among a susceptible host, pathogen and certain environmental conditions (Roberts, 1993; Toranzo *et al*., 2005). Regarding infectious diseases caused by bacteria in fish, Roberts (1993) further highlighted that they are commonly 'stress' related. If stress is repeated, persistent or mismanaged then it will result in an allostatic load or cost, leading to immuno-suppression (McEwen and Wingfield, 2003). Consequently, external stressors often play an important role in disease susceptibility and may play a pivotal role in the progression from exposure to a pathogen to the development of clinical disease. A list of known pathogens causing disease of Nile tilapia is illustrated in Table 1.1. By far the biggest disease problems affecting this industry are due to streptococcal infections (Welker and Lim, 2011).

Table 1.1 List of known pathogens of Nile Tilapia (FAO, 2012; Soto *et al*., 2013a, Soto *et al*., 2013b)

Bacteria		Other	
Disease	Pathogen		Pathogen
Columnaris	*Flavobacterium columnare*	Fungi: Saprolegniasis	*Saprolegnia parasitica*
Edwardsiellosis	*Edwardsiella tarda* and *E. ictaluri*	Metazoan: Monogenetic trematodes	*Dactylogyrus* spp., *Gyrodactylus* spp.
Francisellosis	*Francisella tularensis*	Protozoa: Ciliates	*Ichthyophthirius multifiliis, Trichodina* and others
Motile aeromonas Septicaemia	*Aeromonas hydrophila* and related species		
Streptococcosis	*Streptococcus iniae, S. agalactiae* and *Enterococcus* sp.		
Vibriosis	*Vibrio anguillarum* and other species		

1.3 Streptococcosis in fish

Streptococcosis is a bacterial disease affecting a wide range of fish species globally and is reported to cause considerable fish morbidity and mortality. Severe economic losses caused by this disease have ensued for decades. In 1997 it was estimated that the annual economic loss caused through streptococcal infections exceeded US$150 million worldwide (Shoemaker and Klesius, 1997). More recently large-scale streptococcal outbreaks in China resulted in an reported loss of approximately US$0.4 billion in 2011 (Chen *et al*., 2012).

Streptococcosis infections in fish can be caused by more than one *Streptococcus* species (Austin and Austin, 2007). *Streptococcus* spp. are a large genus of Gram-positive cocci which are non-motile and catalase negative and can be either alpha-, beta- or non-haemolytic. Of the pathogenic *Streptococcus* spp. *S. agalactiae* and *S. iniae* are recognised as the major bacterial pathogens affecting cultured and wild populations of fresh and marine water fish species throughout the world (Agnew and Barnes, 2007; Mian *et al*., 2009). Mortalities are often high with as much as 75% being reported due to *S. iniae* in a commercial hybrid tilapia farm (*Oreochromis niloticus* x *O. aureus*) (Perera and Johnson, 1994). In addition, mortalities exceeding 80% have been reported in red tilapia (*Oreochromi*s spp.) farms infected with *S.*

agalactiae (Zamri-Saad *et al*., 2010). These aetiological agents of streptococcosis are therefore considered as important aquatic pathogens of global veterinary importance.

1.3.1 Classification

Two species of streptococci, *S. shiloi and S. difficile*, were named in 1994 as a result of a bacterial disease outbreak in, what was called at the time, St. Peter's fish (*Oreochromis* spp.) and rainbow trout (*Oncorhynchus mykiss*) in Israel during 1986 (Eldar *et al*., 1994). These were considered as newly described species of fish pathogens causing meningo-encephalitis in cultured fish; *S. difficile* was additionally described as serologically non-typeable (Eldar *et al*., 1994). However, Vandamme *et al*. (1997) demonstrated through whole-cell protein electrophoresis that the type strain of *S. difficile* was indistinguishable from *S. agalactiae* strains when recovered from various host sources. Their genetic relatedness was demonstrated through comparative nucleic sequence analysis using the 16S-23S ribosomal DNA intergenic spacers of *S. difficile* and *S. agalactiae* (Berridge *et al*., 2001). Kawamura *et al*. (2005) also found high levels of genetic similarly between these putative species and suggested that although there are biochemical differences between them, *S. difficile* and *S. agalactiae* are synonyms. In addition, the specific epithet of *S. difficile* was emended by Euzéby (1998) to *S. difficilis*. Similarly, it was also found that *S. shiloi* and *S. iniae* strains were phenotypically identical and through DNA-DNA hybridization the level of homology between strains was 77 – 100%. Thus Eldar *et al*. (1995b) declared that *S. shiloi* should be considered as a junior synonym to *S. iniae.*

Many *Streptococcus* species initially described in the literature were simply portrayed as part of the broad category of *Streptococcus* spp. and not classified as a named subspecies. However, through comparing the biochemical characteristics of isolates and/or using additional identification techniques such as direct fluorescent antibody technique, there has been unification of the classification of strains found in different reports. Consequently, Kitao (1993) highlighted that 'strains described as beta-haemoltyic *Streptococcus* species by Japanese researches should be classified as a subspecies of *S. iniae'*. This includes results obtained from Minami (1979), Kitao *et al*. (1981), Ohnishi and Jo (1981), Nakatsugawa (1983) and Sakai *et al*. (1986). Kitao (1993) further stated 'The *Streptococcus* species described by Robinson and Meyer (1966), Plumb *et al*. (1974) and Rasheed and Plumb (1984) all proved to be the same category, namely non-haemolytic or gamma haemolytic, and Lancefield's B group'. Since *S. agalactiae* is a Group B *Streptococcus* (GBS) (Lancefield, 1933) and identification of this bacterium is partly based on its haemolytic reaction on blood agar and Lancefield grouping (Garcia *et al.*, 2008), the *Streptococcus* species reported by these studies are considered as *S. agalactiae.*

1.3.2 Distribution and host range

The first streptococcal infection in fish was reported in 1958 from cultured rainbow trout (*O. mykiss*) in Japan (Hoshina *et al*., 1958). Since then, several streptococcal infections have been identified worldwide in farmed, wild, freshwater, marine and euryhaline species. In 1976, *S. iniae* was isolated from subcutaneous abscesses in a captive Amazon freshwater dolphin (*Inia geoggrensis*) in the USA (Pier and Madin, 1976). Subsequently, *S. iniae* infections in fish were described in the 1980s (Agnew and Barnes, 2007) and confirmed cases have been reported frequently and persistently worldwide ever since. There are at least 40 documented fish species that have been infected with *S. iniae* (Table 1.2). Although there is a broad host range for this bacterial pathogen, common carp (*Cyprinus carpio*) (Eldar *et al*., 1995a) and channel catfish (*Ictalurus punctatus*) (Shoemaker *et al*., 2001) have been reported to be non-suspectible.

In 1966, Robinson and Meyer reported what is considered to be the first case of Group B *Streptococcus* (*S. agalactiae*) in golden shiners (*Notemigonus crysoleucas*) in the USA. The reports of *S. agalactiae* infections in other fish species are illustrated in Table 1.3 and would suggest that the host range of this pathogen appears more restrictive than that of *S. iniae*. Additionally, several fish species were found to be resistant to infection by intraperitoneal injections of the streptococcal fish pathogen identified by Robinson and Meyer (1966). These species were the mouth buffalo (*Ictiobus cyprinellus*), goldfish (*Carassius auratus*), black crappie (*Pomoxis nigromaculatus*), largemouth bass (*Micropterus salmoides*) and channel catfish.

As shown in Figure 1.2 there are reports of *Streptococcus* infections in fish from numerous countries across the world. *Streptococcus agalactiae* infections have been reported from 21 countries or areas and *S. iniae* infections from 27 countries or areas. *Streptococcus agalactiae* and *S. iniae* are both found in 14 of these countries.

Table 1.2 Fish species reported to have been naturally infected with *Streptococcus iniae*

Common name	Scientific name	Location(s)	Reference
Amago salmon	*Oncorhynchus rhodurus var. macrostomus*	Japan	Kitao, 1993; Ohnishi and Jo, 1981
Ayu salmon	*Plecoglossus altivelius*	Japan	Kitao, 1993; Ohnishi and Jo, 1981
Barramundi	*Lates calcarifer*	Australia	Bromage *et al*., 1999; Creeper and Buller, 2006
		Israel	Kvitt and Colorni, 2004
Barramundi cod	*Cromileptes altivelis*	Australia	Bromage and Owens, 2002
Black margate	*Anisotremus* spp.	The Grenadines	Ferguson *et al*., 2000
Black-saddled grouper	*Epinephelus bleekeri*	China	Zhou *et al*., 2008
Channel catfish	*Ictalurus punctatus*	China	Chen *et al*., 2011
Chubb	*Scaridae* spp.	Barbados	Ferguson *et al*., 2000
Dusky spinefoot	*Siganus fuscescens*	Japan	Sugita, 1995
European seabass	*Dicentrarchus labrax*	Israel	Kvitt and Colorni, 2004; Zlotkin *et al*., 1998
Flat bream	*Rhabdosargus sarba*	China	Zhou *et al*., 2008
Gilthead seabream	*Sparus aurata*	Israel	Zlotkin *et al*., 1998
		Spain	Aamri *et al*., 2010
Gold spot cod	*Epinephalis tauvina*	Australia	Bromage and Owens, 2002
Grunt	*Haemulidae* spp.	Barbados	Ferguson *et al*., 2000; Kvitt and Colorni, 2004
Hybrid nile x blue tilapia	*Oreochromis niloticus* x *O. aureus*	USA	Perera and Johnson, 1994
Hybrid striped bass (Sunshine bass)	*Morone chrysops* x *M. saxatilis*	USA	Shoemaker *et al*., 2001; Stoffregen *et al*., 1996

Japanese flounder (Olive flounder)	*Paralichthys olivaceus*	Japan	Kitao, 1993; Nguyen *et al*., 2002
		Korea	Nho *et al*., 2009
Jacopever	*Sebastes schlegeli*	Japan	Kitao, 1993; Sakai *et al*., 1986
Lizardfish	*Synodus variegatus*	Israel	Colorni *et al*., 2002; Kvitt and Colorni, 2004
Lyretail grouper	*Variola louti*	Israel	Kvitt and Colorni, 2004
Muddy grouper	*Epinephelus bruneus*	China	Zhou *et al*., 2008
Parrot fish	*Sparisoma aurofrenatum* and *S. viride*	Barbados and The Grenadines	Ferguson *et al*., 2000
	Sparisoma aurofrenatum	Caribbean	Keirstead *et al*., 2013
Pompano	*Trachinotus ovatus*	China	Zhou *et al*., 2008
Princess parrotfish	*Scarus taeniopterus*	Caribbean	Keirstead *et al*., 2013
Puffer fish	*Arothron hispidus*	Australia	Bromage and Owens, 2002
Rabbit fish	*Siganus* spp.	Australia	Bromage and Owens, 2002
		Bahrain	Yuasa *et al*., 1999
		China	Zhou *et al*., 2008
		Israel	Zlotkin *et al*., 1998
		Singapore	Foo *et al*.,1985
Rainbow shark	*Epalzeorhynchos erythrurus*	USA	Russo *et al*., 2006
Rainbow trout	*Oncorhynchus mykiss*	Italy	Ghittino *et al*., 2003
		Iran	Erfanmanesg *et al*., 2012
		Israel	Eldar and Ghittino, 1999; Kvitt and Colorni, 2004
		Japan	Kitao, 1993; Kitao *et al*., 1981
Red drum	*Sciaenops ocellatus*	China	Shen *et al*., 2005; Zhou *et al*., 2008
		Israel	Kvitt and Colorni, 2004
Red hind	*Epinephelus guttatus*	Caribbean	Keirstead *et al*., 2013
Red tail black shark	*Epalzeorhynchos bicolor*	USA	Russo *et al*., 2006
Red porgy	*Pagrus pagrus*	Spain	Aamri *et al*., 2010
Red snapper	*Lutjanus erythropterus*	China	Zhou *et al*., 2008
	Lutjanus campechanus	Caribbean	Keirstead *et al*., 2013
Silver bream	*Acanthopagrus australis*	Australia	Bromage and Owens, 2002
Spine foot	*Siganus rivulatus*	Israel	Zlotkin *et al.*, 1998
Spotted silver scat	*Scatophagus argus*	China	Zhou *et al*., 2008
Striped piggy grunt	*Pomadasys stridens*	Israel	Colorni *et al*., 2002; Kvitt and Colorni, 2004
Striped bass	*Morone saxatilis*	Israel	Kvitt and Colorni, 2004
Tilapia	*Oreochromis* spp.	Brazil	Figueiredo *et al*., 2012
		Canada	Dodson *et al*., 1999
		China	Zhou *et al*., 2008
		Colombia	Conroy, 2009
		Ecuador	Sheehan, 2009
		Honduras	Sheehan, 2009
		Indonesia	Sheehan, 2009
		Israel	Eldar *et al*., 1994; Kvitt and Colorni, 2004
		Japan	Kitao, 1993; Kitao *et al*., 1981
		Peru	Conroy, 2009
		Philippines	Sheehan, 2009
		Taiwan	Eldar *et al*., 1994
		Thailand	Sheehan, 2009
		Uruguay	Conroy, 2009
		USA	Bowser *et al*., 1998; Shoemaker *et al*., 2001
		Venezuela	Conroy, 2009
		Vietnam	Sheehan, 2009
Threeband sweetlips	*Plectorhynchus cinctus*	China	Zhou *et al*., 2008
Turbot	*Scophthalmus maximus*	China	Zhan *et al.*, 2009
Yellowtail	*Seriola quinqueradiata*	Japan	Kaige *et al*., 1984; Kitao, 1993; Minami, 1979
Yellow seabream	*Acanthopagrus latus*	China	Zhou *et al*., 2008
Yellowtail snapper	*Ocyurus chrysurus*	Barbados	Ferguson *et al*., 2000; Kvitt and Colorni, 2004
		Caribbean	Keirstead *et al*., 2013

Table 1.3 Fish species reported to have been naturally infected with *Streptococcus agalactiae*

Common name	Scientific name	Location(s)	Reference
Atlantic croaker	*Micropogon undulatus*	Gulf of Mexico	Plumb *et al*., 1974
Bartail flathead	*Platycephalus indicus*	Kuwait	Jafar *et al*., 2009
Bluefish	*Pomatomus saltatrix*	USA	Baya *et al*., 1990
Catfish	*Arius thalassinus*	Australia	Bowater *et al*., 2012
		Kuwait	Al-Marzouk, 2005; Jafar *et al*., 2009
Doctor fish	*Garra rufa*	UK	Verner-Jeffreys *et al*., 2012
Eastern shovelnose ray	*Aptychotrema rostrata*	Australia	Bowater *et al*., 2012
Estuary rays	*Dasyatis fluviorum*	Australia	Bowater *et al*., 2012
Golden pompano	*Trachinotus blochii*	Malaysia	Amal *et al*., 2012
Golden ram	*Mikrogeophagus ramirezi*	Australia	Delannoy *et al*., 2013
Golden shiners	*Notemigonus crysoleucas*	n/a	Robinson and Meyer, 1966
Giant Queensland grouper	*Epinephelus lanceolatus*	Australia	Bowater *et al*., 2012
Gilthead seabream	*Sparus auratus L.*	Kuwait	Evans *et al*., 2002
Grey mullet	*Mugus cephalus*	Israel	Eldar *et al*., 1995a
Gulf killifish	*Fundulus grandis*	USA	Rasheed and Plumb, 1984
Gulf menhaden	*Brevoortia patronus*	Gulf of Mexico	Buller, 2009; Plumb *et al*., 1974
Hybrid Amazon catfish	*Pseudoplatystoma fasciatum x Leiarius marmoratus*	Brazil	Godoy *et al*., 2013
Javelin grunter	*Pomadasys kaakan*	Australia	Bowater *et al*., 2012
Mangrove whipray	*Himantura granulata*	Australia	Bowater *et al*., 2012
Mullet	*Liza klunzingeri*	Kuwait	Al-Marzouk, 2005; Evans *et al*., 2002
Pinfish	*Lagodon rhomboide*	Gulf of Mexico	Plumb *et al*., 1974
Rainbow trout	*Oncorhynchus mykiss*	Iran	Pourgholam *et al*., 2011
		Israel	Eldar *et al*., 1994
Rosy barb	*Puntius conchonius*	Australia	Delannoy *et al*., 2013
Sea catfish	*Arius felis*	Gulf of Mexico	Plumb *et al*., 1974
Sea trout	*Cynoscion regalis*	USA	Baya *et al*., 1990
Silver trout	*Cynoscion nothus*	Gulf of Mexico	Plumb *et al*., 1974
Silver pomfret	*Pampus argenteus*	Kuwait	Duremdez *et al*., 2004
			Azad *et al*., 2012
Silvery croaker	*Otolithes argenteus*	Kuwait	Al-Marzouk, 2005
Spot	*Leiostomus xanthurus*	Gulf of Mexico	Plumb *et al*., 1974
Stingray	*Dasyatis* spp.	Gulf of Mexico	Plumb *et al*., 1974
Striped bass	*Morone saxatilis*	USA	Baya *et al*., 1990
	Morone saxatilis x M. chrysops	Israel	Garcia *et al*., 2008
Striped grunt	*Rhonciscus stridens*	Kuwait	Al-Marzouk, 2005
Striped mullet	*Mugil cephalus*	Gulf of Mexico	Plumb *et al*., 1974
Striped piggy grunt	*Pomadasys stridens*	Kuwait	Jafar *et al*., 2009
Tilapia	*Oreochromis* spp.	Belgium	Delannoy *et al*., 2013
		Brazil	Mian *et al*., 2009; Salvador *et al*., 2005
		China	Chen *el al*., 2012; Sheehan, 2009
		Colombia	Conroy, 2009; Hernández *et al*., 2009
		Costa Rica	Delannoy *et al*., 2013
		Ecuador	Sheehan, 2009
		Honduras	Sheehan, 2009
		Indonesia	Sheehan, 2009
		Israel	Eldar *et al.,* 1994; Eldar *et al*., 1995a
		Malaysia	Abuseliana *et al*., 2010; Musa *et al*., 2009
		Mexico	Sheehan, 2009
		Philippines	Sheehan, 2009
		Thailand	Delannoy *et al*., 2013; Suanyuk *et al*., 2008
		USA	Conroy, 2009
		Vietnam	Delannoy *et al*., 2013; Sheehan, 2009
Ya-Fish	*Schizothorax prenanti*	China	Geng *et al*., 2012
Yellowtail	n/a	Japan	Eldar *et al*., 1994

[n/a] information not available

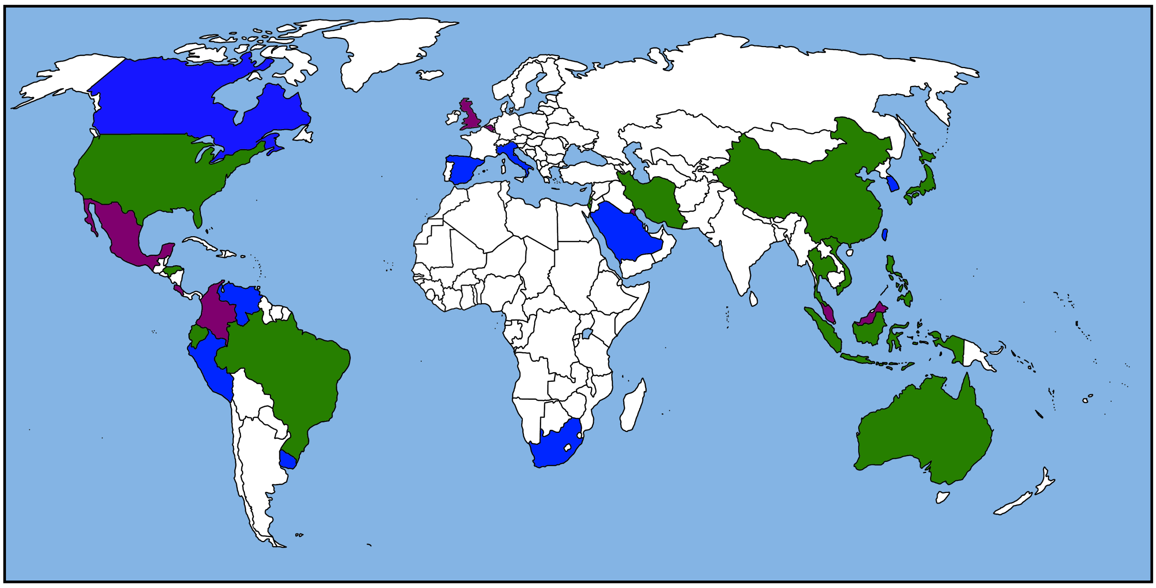


Figure 1.2 Global distribution of reported cases of [blue] *Streptococcus iniae* [purple] *Streptococcus agalactiae* [green] both *S. agalactiae* and *S. iniae* infections in fish. Data used from Table 1.2 and 1.3. *Streptococcus iniae* infections from fish in South Africa and Saudi Arabia taken from Austin and Austin (2007).

1.3.3 Factors influencing outbreaks of streptococcosis

Environmental conditions can have considerable impact on the occurrence of infectious diseases caused by bacterial pathogens in fish. Firstly, certain environmental conditions can increase the pathogenic ability of the bacterium by enhancing their ability to adhere, invade and colonise a host. Secondly, suboptimum environmental conditions can cause a stress response within the fish host, compromising their immune system and making them more vulnerable to infections. An outbreak of *Streptococcus* is often initiated through host-environment interactions, with the main predisposition being external stressors. Increased susceptibility to *Streptococcus* has been associated with suboptimum salinities (Chang and Plumb, 1996), temperatures (Al-Marzouk *et al*., 2005; Chang and Plumb, 1996; Perera *et al*., 1997; Rodkhum *et al*., 2011), pH (Perera *et al*., 1997), dissolved oxygen (Evans *et al*., 2003) and stocking densities (Shoemaker *et al*., 2000).

1.3.4 Clinical signs of disease

Clinical signs of disease can be a combination of behavioural abnormalities with changes in external and internal organs in fish. These can often be variable and dependent on host species, fish age and stage of the disease (Toranzo *et al*., 2005). Fish infected with *S. agalactiae* or *S. iniae* show similar disease signs that are also comparable to other bacterial infections.

The most commonly reported clinical signs associated with streptococcal infections in fish include: exophthalmia (unilateral or bilateral), corneal opacity, intra ocular haemorrhages, lethargy, loss of appetite, loss of orientation or sudden death (Bercovier *et al*., 1997; Bromage and Owens, 2002; Duremdez *et al*., 2004; Eldar *et al*., 1995a; Evans *et al*., 2002; Perera *et al*., 1994; Perera *et al*., 1997). Additional observations include fluid accumulation in the peritoneal cavity, darkening of the skin, abdominal swelling and an enlarged spleen (Duremdez *et al*., 2004; Eldar and Ghittino, 1999; Filho *et al*., 2009; Perera *et al*., 1994). Additionally, haemorrhage in the integumental and muscoskeletal system may be observed; with reddening most frequent around the mouth, anus and fins (Evans *et al*., 2002; Perera *et al*., 1994; Perera *et al*., 1997). Behavioural changes include various signs of disorientation, erratic swimming, swimming and whirling at the water surface, 'C'-shaped body curvature and head-up or tail-up swimming (Bromage and Owens, 2002; Evans *et al*., 2000; Evans *et al*., 2002; Filho *et al*., 2009). Some reports have stated that *S. agalactiae* infection may result in the operculum becoming transparent, referred to as a 'window to the gills' (Evans *et al*., 2002), long mucoid faecal casts (Pasnik *et al*., 2005) and enlarged absecess-like swellings in the skin (Bowater *et al*., 2012). A comparison between reported clinical signs of disease from natural and experimental *S. agalactiae* and *S. iniae* infections are illustrated in Table 1.4.

Table 1.4 Clinical signs of disease reported in fish with *Streptococcus agalactiae* or *Streptococcus iniae* infections

	S. iniae	Reference	*S. agalactiae*	Reference
Behavioural	Abnormal swimming/ loss of orientation	Aamri *et al*., 2010	Erratic swimming	Ye *et al*., 2011; Zamri-Saad *et al*., 2010
	Lethargy	Aamri *et al*., 2010;	**Lethargy**	Abuseliana *et al*., 2010
	Loss of appetite	Chen *et al*., 2011	**Loss of appetite**	Abuseliana *et al*., 2010
	Increased ventilation rate	Bromage *et al*., 1999	**Grouping at aquarium bottom**	Abuseliana *et al*., 2010
External	Darkening of the skin	Eldar and Ghittino, 1999; Yuasa *et al*., 1999	Discolouration	Zamri-Saad *et al*., 2010.
	Cornea opacity	Colorni *et al*., 2002; Eldar and Ghittino, 1999	Corneal opacity	Ye *et al*., 2011; Zamri-Saad *et al*., 2010
	Skin lesion	Colorni *et al*., 2002	Skin ulcers/lesions	Bowater *et al*., 2012
	Anal inflammation	Eldar and Ghittino, 1999	**Anal swelling**	Rodkhum *et al*., 2011
	External petechial haemorrhages	Chen *et al*., 2011	External haemorrhages	Evans *et al*., 2002; Wang *et al*., 2013
	Proximal margins of fins	Perera and Johnson, 1994	**Fin rot**	Abuseliana *et al*., 2010
	Diarrhoea	Eldar and Ghittino, 1999	**Long faecal casts**	Pasnik *et al*., 2009
	Exophthalmos	Aamri *et al*., 2010; Zhou *et al*., 2008	Exophthalmos	Ye *et al*., 2011; Zamri-Saad *et al*., 2010
	Sudden death	Aamri *et al*., 2010	Sudden death	Zamri-Saad *et al*., 2010.
	Anorexia	Aamri *et al*., 2010	**Anorexia**	Filho *et al*., 2009
	Abdominal swelling	Perera and Johnson, 1994	Abscess-like swellings in skin	Bowater *et al*., 2012
	Intraocular haemorrhage	Eldar and Ghittino, 1999	Clear opercula	Evans *et al*., 2002
			Corneal perforation	Bowater *et al*., 2012
			'C'-shaped body curvature	Evans *et al*., 2002; Hernández *et al*., 2009
Internal	Intestines filled with yellow fluid	Chen *et al*., 2011	Yellow intestinal mucus	Wang *et al*.,2013
	Splenomegaly	Eldar and Ghittino, 1999; Zhou *et al*., 2008	Splenomegaly	Bowater *et al*., 2012
	Hepatomegaly and pale liver	Perera *et al*., 1994; Yuasa *et al*., 1999	Hepatomegaly and **pale liver**	Geng *et al*., 2012; Wang *et al*., 2013
	Ascites	Chen *et al*., 2011; Yuasa *et al*., 1999	Ascites	Evans *et al*., 2002; Ye *et al*., 2011
	Enlarged and pale kidney	Perera and Johnson, 1994; Zhou *et al*., 2008	**Enlarged kidney**	Rodkhum *et al*., 2011
	Turgid gallbladder	Zhou *et al*., 2008	Enlarged gallbladder	Wang *et al*.,2013
	Haemorrhagic septicaemia	Aamri *et al*., 2010	Haemoperitoneum	Bowater *et al*., 2012
	Haemorrhage of gills	Colorni *et al*., 2002	White fibrinous exudate coving the heart	Hernández *et al*., 2009
	Dilated intestine	Eldar and Ghittino, 1999	**Petechial haemorrhage at liver**	Rodkhum *et al*., 2011
	Intracranial oedema	Eldar and Ghittino, 1999	**Epicardial opacity**	Filho *et al*., 2009
	Internal haemorrhage	Colorni *et al*., 2002; Eldar and Ghittino, 1999;	Congestion of viscera	Zamri-Saad *et al*., 2010.
	Gill pallor	Eldar and Ghittino, 1999		

All clinical signs of disease were from natural disease outbreaks. **Bold red** lettering represents clinical signs apparent during experimental challenges within the literature.

1.3.5 Pathology

Chen *et al*. (2007) conducted a comprehensive comparative histopathological investigation into tilapia infected naturally and experimentally with either *S. agalactiae* or *S. iniae.* They found that both pathogens can cause pericarditis, epicarditis, myocarditis, endocarditis and meningitis. Additionally, *S. agalactiae* infected tilapia had large numbers of cocci present in tissues and in the circulation, which were not observed in *S. iniae* infected tilapia. The authors also suggested that tilapia only develop a chronic form of *S. iniae* during a natural disease outbreak as the fish are more effective in controlling the infection, whereas this was not the case for natural infections of *S. agalactiae*. For experimental *S. iniae* infections lymphohistiocytic, leptomeningitis, meningoencephalitis, encephalitis and meningitis were also described in infected tilapia (Baums *et al*., 2013).

Hernández *et al*. (2009) suggested that *S. agalactiae* has a predilection for organs such as the brain, eyes and heart. Zamri-Saad *et al*. (2010) also noted that during a natural disease outbreak of *S. agalactiae* the liver, spleen and kidney showed the presence of marked congestion and the endothelial cells lining major blood vessels for the liver and spleen were swollen and vacuolated. Histological findings from experimental and natural disease outbreaks of *S. agalactiae* have previously been described by Abuseliana *et al*. (2010), Azad *et al*. (2012) and Filho *et al*. (2009).

1.3.6 Diagnosis

1.3.6.1 Culture and biochemical tests

Buller (2009) stated that primary identification tests for a bacterium should include microscopic examination of smears (Gram stain), catalase, oxidase, presence of haemolysis, motility and growth on agar plates. *Streptococcus agalactiae* and *S. iniae*, as described by Austin and Austin (2007), produce colonies on Brain Heart Infusion Agar (BHIA) that are 1 mm in diameter and are non-pigmented after an aerobic incubation at 30 °C for 24 hours. Cells of both species contain Gram-positive cocci, which are fermentative, catalase-negative, oxidase-negative and non-motile. *Streptococcus iniae* shows complete beta-haemolysis on sheep blood agar (SBA) (Buller, 2009) whereas variations in haemolysis have been observed between *S. agalactiae* strains (Sheehan, 2009). Consequently, isolates of *S. agalactiae* have been differentiated into two distinct clusters, known as Biotype 1 and Biotype 2, which differ in their biochemical and phenotypic characteristics. Biotype 1 isolates are beta-haemolytic whereas typically non-beta-haemolytic *S. agalactiae* are referred to as Biotype 2. An epidemiological study by Sheehan (2009) conducted in 13 tilapia-producing countries found that 26% of all streptococcal isolates from tilapia were *S. agalactiae* Biotype 1, 56% were *S. agalactiae* Biotype 2 and 18% were identified as *S. iniae.*

Biochemical profiles are determined as part of the secondary identification tests to identify an organism to species level (Buller, 2009). These tests may be conducted through conventional biochemical methods or through commercial identification systems such as the API identification system (bioMérieux, Marcy l'Etoile, France). Streptococcal bacteria can also be serologically classified using the Lancefield grouping (20 serotypes A-H, K-V) which is based on the polysaccharide antigens of cell wall carbohydrates. There are several commercially available latex agglutination immunological assays for the detection of these groups; *S. agalactiae* belongs to Lancefield Group B whereas *S. iniae* does not belong to any Lancefield group.

Since the late 1960s, miniaturized identification systems have been utilised in microbiology laboratories and are considered to be 'very accurate for the more common species and provide quick test results in a cost-effective manner' (Janda and Abbott, 2002). However, there can be discrepancies in biochemical results observed from commercial systems and those from conventional tests making identification and classification of bacteria problematic. Research demonstrated that BioMerieux Vitek, ATB Expression system (Lau *et al.*, 2003; Lau *et al*., 2006) and Microscan (Facklam *et al.*, 2005) were unable to identify *S. iniae* from human cases due to the absence of *S. iniae* catalogues in the corresponding databases. For aquatic *S. iniae* isolates only 76% could be identified using the Biolog GP microplate panels and Microlog database (Roache *et al.*, 2006). Furthermore, phenotypic characteristics can be unstable as expression may be dependent upon an array of environmental factors such as temperature and pH levels (Janda and Abbott, 2002). For example, Vandamme *et al.* (1997) found that there was a difference in the activity of beta-glucuronidase and the hydrolysis of hippurate within the *S. difficile* type strain when incubating API 20 STREP strips at different temperatures. Intraspecific variants (including serotypes) of bacterial isolates have also been shown to differ biochemically, which may consequently influence results obtained from commercial identification systems. *Streptococcus iniae* has two distinct serotypes that differ in their ability to react with arginine dihydrolase (ADH) and ribose (Agnew and Barnes, 2007). Variation in ADH activity in *S. iniae* appears to be an artefact of the commercial API 20 strep kit as it was found that through a conventional direct assay procedure the ADH activity was always positive (Barnes and Ellis, 2003). These authors also investigated the effect of bacterial cell concentration on API 20 strep results and found that culture dilution could affect readings made from this commercial kit (Barnes and Ellis, 2003).

The lack of robust comparative data from commercial identification systems limits their usefulness for aquatic disease diagnosis. Consequently, current identification procedures regularly use molecular techniques alongside commercial bacterial identification systems.

1.3.6.2 Molecular characterisation

Several molecular techniques have been utilised to complement conventional diagnostic procedures for the identification of *S. agalactiae* and *S. iniae*. Such techniques include: randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and whole genome DNA-DNA hybridisation. Klesius *et al*. (2006) developed a monoclonal antibody-based indirect fluorescent antibody technique for the detection and identification of *S. iniae*. Suebsing *et al*. (2013) also established a colorimetric loop-mediated isothermal amplification (LAMP) assay with pre-addition of calcein for the visual detection of *S. agalactiae* and *S. iniae.* However, the standard molecular technique for identifying bacteria at the species level is through a polymerase chain reaction (PCR).

A PCR-based method allows for rapid identification, has a high sensitivity and can be used with minute amounts of samples. The identification procedure is based on the selective amplification of rRNA gene fragments, which are highly conserved within the bacteria (Pheuktes *et al*., 2001). In particular, the characterisation of the 16S rRNA gene has been widely utilised for the identification of species, genera and families of bacteria (Amann *et al*., 1995; Gürtler and Stanisich, 1996). However, there can occasionally be very little sequence variation observed between 16S rRNA genes of closely related microorganisms (Barry *et al*., 1991), which may lead to misidentification through lack of resolution or specificity. The PCR primer sets employed by Zlotkin *et al*. (1998) were based on the amplification of the *S. iniae* 16S rRNA gene sequence however, similarly sized amplification products have been observed for both *S. iniae* and *S. difficilis* strains (Mata *et al*., 2004). Mata *et al*. (2004) claimed that the high genetic relatedness between these two bacterial species may explain this non-specific amplification. Consequently, an alternative strategy has been developed that uses the 16S - 23S rRNA intergenic spacer region; this is suggested to have considerable variation between species in both the length and sequence of this region (Barry *et al*., 1991; Gürtler and Stanisich, 1996). The reason for this is that the non-functional spacer regions are considered to be less evolutionarily constrained, as it is under minimal selective pressure during evolution, and consequently varies more extensively among closely related bacterial species than the 16S rRNA gene (Barry *et al*., 1991).

Other genes have also been used as biomarkers during PCR assays such as the lactate oxidase-encoding gene (*lctO*) and the chaperonin 60 gene (Cpn60) for the identification of *S. iniae* (Gibello *et al*., 1999; Goh *et al*., 1998; Mata *et al*., 2004). Although the *lctO* gene can be found in other bacterial species, a specific primer combination has been developed that produces a single amplification product which provides specific detection and identification for *S. iniae* (Mata *et al*., 2004). This target gene has also been integrated into a duplex-PCR for the simultaneous detection of *S. agalactiae* and *S. iniae* (Rodkhum *et al*., 2011). However, due to the sensitivity of PCR assays, minor contamination in samples can lead to misdiagnosis (Phuektes *et al*., 2001). Therefore, it is important that definitive bacterial identification is based on biochemical and molecular techniques.

1.3.7 Disease prevention, control and treatment

Prevention and control of disease is a multifactorial process and thus requires an integrated approach to health management (Wendover, 2009). A systemic diagram giving an overview of such management strategies is shown in Figure 1.3. Preventative measures include reducing fish densities, ensuring good water quality, diligent removal of dead/moribund fish, implementing stringent bio-security protocols and minimising ‘stressors’ such as fish handling and transportation.

The supplementation of fish feed with substances such as herbal additives and vitamins have shown to significantly increase survival rates during a *Streptococcus* outbreak. Examples of such substances include vitamin E (Lim *et al*., 2010), thyme, rosemary, fenugreek (Yilmaz *et al*., 2013) and *Sophora flavescens* (Wu *et al*., 2013). The aim of dietary supplements is to act as immunostimulants, enhancing fish immunity and thus disease resistance. These can also be incorporated alongside vaccination programs to promote immune response in fish and consequently amplify protection against *Streptococcus* pathogens (Salvador *et al*., 2012).

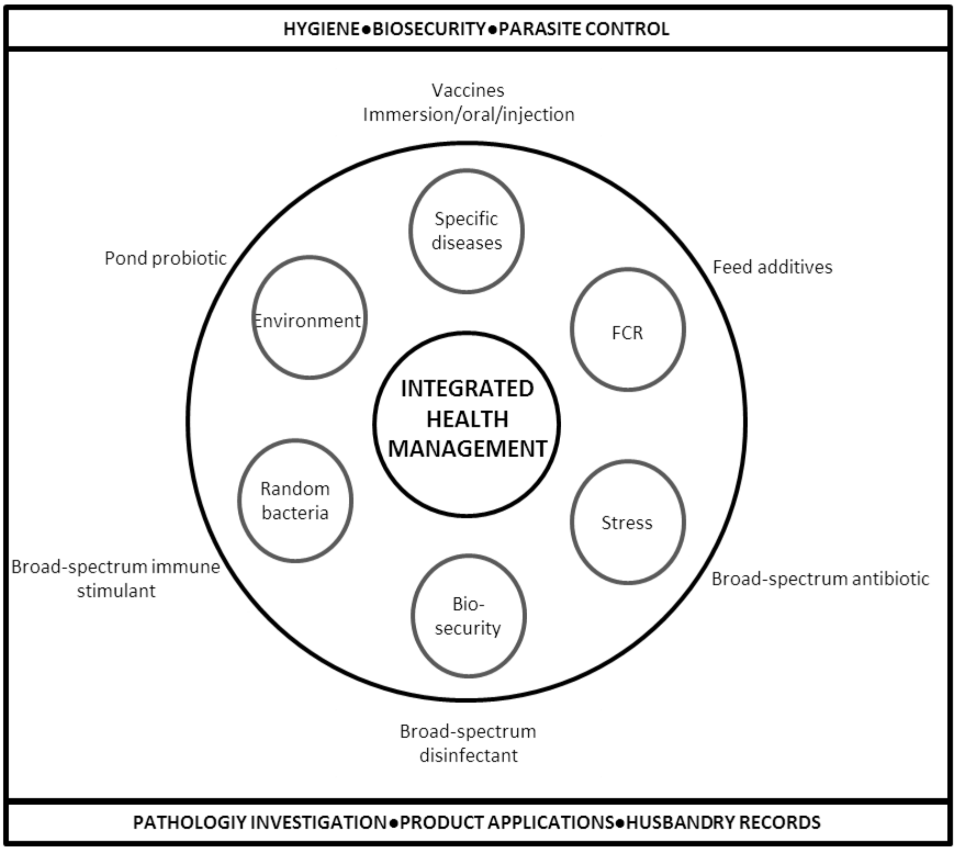


Figure 1.3 Integrated health management for effective prevention and control of disease. Modified from Wendover (2009). [FCR] Feed conversion ratio.

The development and application of vaccines against *Streptococcus* infections has also been widely researched in an attempt to prevent disease outbreaks. Experimental *S. agalactiae* vaccines composed of [a] concentrated extracellular products (ECP) (Evans *et al*., 2004) [b] formalin-killed whole cells (Evans *et al*., 2004) [c] bacterial antigens (Sheehan, 2009) and [d] live attenuated bacteria (Pridgeon and Klesius, 2013) have all been described. Before 2009 none of the vaccines developed for *S. agalactiae* had been commercialised internationally (Sheehan, 2009). More recently, Merck Animal Health Inc developed a commercially available oil adjuvanted injection vaccine, AQUAVAC® Strep Sa, however this only combats *S. agalactiae* Biotype 2 infections and is only for use in fish > 15 g (Pridgeon and

Klesius, 2013). This means that younger fish remain vulnerable to infection. Work from Pridgeon and Klesius (2013) does show potential for future vaccines that could provide a broader protection against infections caused by *S. agalactiae* for fish 3 – 5 g and 15 – 20 g. However, these authors do highlight that developing a polyvalent *S. agalactiae* vaccine that will protect against all *S. agalactiae* strains that can cause streptococcosis will be nearly impossible.

Under experimental conditions, *S. iniae* vaccines in the form of subunit vaccines, DNA vaccines and attenuated live vaccines have been tested (Sun *et al*., 2013). Vaccines such as these have not been commercialised internationally. Other vaccine trials include whole-cell formalin inactivated *S. iniae*, killed bacterins supplemented with ECP and autogenous vaccines (Agnew and Barnes, 2007). There are some commercially available *S. iniae* vaccines such as Norvax® Strep *Si* (Intervet International B.V.) and AQUAVAC™ GARVETIL™ (Intervet/Schering-Plough Animal Health) but these are not approved in all countries and there are no licensed *S. iniae* vaccines in China (Sun *et al*., 2013). Different methods of vaccination have also been investigated. Vaccine administration has been tried through intraperitoneal (i.p.) injection (Evans *et al*., 2004), intramuscular (i.m.) injection (Agnew and Barnes, 2007) bath immersion (Evans *et al*., 2004), spraying (Noraini *et al*., 2013) and orally through incorporating fish feed (Shoemaker *et al*., 2006).

In intensive aquaculture disease occurrence will be inevitable (Wendover, 2009). Integrated health management is based on adopting preventative disease strategies and utilising responsible treatment techniques. This approach aims to guarantee food safety, enhance survival rates during a disease outbreak and allow consistent production (Wendover, 2009). The main treatment strategy applied during *Streptococcus* outbreaks is the oral administration of antibiotics (Figueiredo *et al*., 2012). Within a laboratory environment, feed medicated with amoxicillin has been shown to significantly increase the survival of sunshine bass (*Morone chrysops* x *M. saxatilis*) (Darwish and Ismaiel, 2003) and tilapia (*O. aureus*) (Darwish and Hobbs, 2005) during an *S. iniae* infection. Within a natural disease outbreak the use of erythromycin and oxytetracycline has been shown to lower mortality rates in barramundi (*Lates calcarifera*) (Crepper and Buller, 2006). *Streptococcus agalactiae* and *S. iniae* recovered from natural disease outbreaks are often tested for antibiotic sensitivity. Results from such tests will influence which antibiotic is subsequently used if the outbreak persists. The use of certain antibiotics will be restricted due to their licensed availability in different countries or if fish are destined for human consumption. These limitations can be very stringent; in Brazil for example only florfenicol is approved for use in tilapia farms (Figueiredo *et al*., 2012). Pridgeon and Klesius (2013) also state that there are only three antibiotics currently approved for use in aquaculture to control *S. agalactiae* infections, these include oxytetracycline (Terramycin), sulfadimethoxine (Romet-30), and florfenicol (Aquaflor).

Testing antibiotic sensitivity can also help identify patterns of emerging resistance and consequently treatment policies can be adapted as required. Furthermore, the sensitivity to some antibiotics has been shown to be variable between different isolates within a species (Wang *et al*., 2013). The reported susceptibility of *S. agalactiae* and *S. iniae* to various antimicrobial agents is illustrated in Tables 1.5 and 1.6. Yet, there are several noticeable limitations to using antibiotics during a disease outbreak. For instance, they are expensive to use as medicated feed, it is common that diseased fish do not feed so administration of antibiotics is challenging, drug residues are of concern if fish are to be sold as human food and there is a risk of pathogens developing antibiotic resistance (Agnew and Barnes, 2007; Pridgeon and Klesius, 2013).

Table 1.5 Sensitivity and resistance of *Streptococcus iniae* isolates recovered from diseased tilapia to various antibiotics

	Antibiotic	Reference
Sensitive	Ampicillin	Eldar *et al*., 1994; Suanyuk *et al*., 2010
	Bacitracin	Perera and Johnson, 1994
	Cefalotin	Eldar *et al*., 1994
	Cefuroxime	Eldar *et al*., 1994
	Cephalothin	Perera and Johnson, 1994
	Chloramphenicol	Eldar *et al*., 1994; Perera and Johnson, 1994; Suanyuk *et al*., 2010
	Ciprofloxacin	Eldar *et al*., 1994
	Clindamycin	Perera and Johnson, 1994
	Erythromycin	Eldar *et al*., 1994; Perera and Johnson, 1994; Suanyuk *et al*., 2010
	Florfenicol	Figueiredo *et al*., 2012
	Fusidic acid	Eldar *et al*., 1994
	Gentamicin	Perera and Johnson, 1994; Suanyuk *et al*., 2010
	Methicillin	Eldar *et al*., 1994; Perera and Johnson, 1994
	Mezlocillin	Eldar *et al*., 1994
	Neomycin	Perera and Johnson, 1994
	Nitrofurantoin	Eldar *et al*., 1994; Suanyuk *et al*., 2010
	Nitrofurazone	Perera and Johnson, 1994
	Norfloxacine	Suanyuk *et al*., 2010
	Ofloxacin	Eldar *et al*., 1994
	Oxytetracycline	Perera and Johnson, 1994; Suanyuk *et al*., 2010
	Penicillin/Penicillin G	Eldar *et al*., 1994; Perera and Johnson, 1994; Suanyuk *et al*., 2010
	Tetracycline	Eldar *et al*., 1994; Perera and Johnson, 1994
	Sulfamethoxazole trimethoprim	Eldar *et al*., 1994; Perera and Johnson, 1994; Suanyuk *et al*., 2010
	Trimethoprim	Suanyuk *et al*., 2010
	Vancomycin	Eldar *et al*., 1994
Resistant	Amikacin	Eldar *et al*., 1994
	Ampicillin	Perera and Johnson, 1994
	Colistin	Eldar *et al*., 1994
	Furazolidone	Perera and Johnson, 1994
	Gentamicin	Eldar *et al*., 1994
	Nalidixic acid	Eldar *et al*., 1994; Suanyuk *et al*., 2010
	Oxolinic acid	Suanyuk *et al*., 2010

Table 1.6 Sensitivity and resistance of *Streptococcus agalactiae* isolates recovered from diseased tilapia to various antibiotics

	Antibiotic	Reference
Sensitive	Ampicillin	Abuseliana *et al*., 2010; Eldar *et al*., 1994; Jantawan *et al*., 2007; Musa *et al*., 2009
	Amoxicillin	Abuseliana *et al*., 2010; Jantawan *et al*., 2007; Musa *et al*., 2009;
	Cefalotin	Eldar *et al*., 1994; Jantawan *et al*., 2007
	Cefoxitin	Wang *et al*., 2013
	Cefuroxime	Eldar *et al*., 1994
	Chloramphenicol	Abuseliana *et al*., 2010; Jantawan *et al*., 2007; Musa *et al*., 2009
	Ciprofloxacin	Eldar *et al*., 1994; Jantawan *et al*., 2007
	Doxycycline	Jantawan *et al*., 2007
	Enrofloxacin	Jantawan *et al*., 2007
	Erythromycin	Abuseliana *et al*., 2010; Jantawan *et al*., 2007; Musa *et al*., 2009
	Fosfomycin	Musa *et al*., 2009
	Flumequine	Musa *et al*., 2009
	Fusidic acid	Eldar *et al*., 1994
	Gentamicin	Abuseliana *et al*., 2010
	Kanamycin	Musa *et al*., 2009
	Lincomycin	Abuseliana *et al*., 2010; Musa *et al*., 2009
	Methicillin	Eldar *et al*., 1994
	Mezlocillin	Eldar *et al*., 1994
	Nalidixic acid	Musa *et al*., 2009
	Nitrofurantoin	Eldar *et al*., 1994; Musa *et al*., 2009
	Novobiocin	Musa *et al*., 2009
	Ofloxacin	Eldar *et al*., 1994
	Oleandomycin	Musa *et al*., 2009
	Oxolinic acid	Musa *et al*., 2009
	Oxytetracycline	Jantawan *et al*., 2007
	Penicillin	Eldar *et al*., 1994
	Rifampicin	Abuseliana *et al*., 2010
	Spiramycin	Musa *et al*., 2009
	Sulfamethoxazole trimethoprim	Abuseliana *et al*., 2010; Eldar *et al*., 1994; Jantawan *et al*., 2007
	Tetracycline	Abuseliana *et al*., 2010; Eldar *et al*., 1994
	Vancomycin	Abuseliana *et al*., 2010; Eldar *et al*., 1994
Resistant	Amikacin	Abuseliana *et al*., 2010
	Colistin	Eldar *et al*., 1994
	Gentamicin	Eldar *et al*., 1994
	Kanamycin	Musa *et al*., 2009
	Nalidixic acid	Jantawan *et al*., 2007; Musa *et al*., 2009
	Neomycin	Abuseliana *et al*., 2010; Jantawan *et al*., 2007
	Oleandomycin	Musa *et al*., 2009
	Oxolinic acid	Jantawan *et al*., 2007; Musa *et al*., 2009
	Polymyxin B	Jantawan *et al*., 2007
	Spiramycin	Musa *et al*., 2009
	Sulfadimethoxazole	Jantawan *et al*., 2007; Musa *et al*., 2009

1.4 The theory of disease

Koch's postulates have been referenced for over 100 years for evaluating the causal relationship between specific microorganisms to an associated clinical disease (Evans, 1976). The criteria set out by this concept are as follows (Madigen and Martinko, 2006): [1] The organism must always be present in animals suffering from the disease and should not be present in healthy individuals. [2] The organism must be cultivated in a pure culture away from the animal body. [3] Such a pure culture, when inoculated into susceptible animals, must initiate the characteristics disease symptoms. [4] The organism must be reisolated from these experimental animals and cultured again in the laboratory, after which it should still be the same as the original organism.

Although these were derived from work on infectious diseases, such as anthrax and tuberculosis, they have been applied to a plethora of other diseases. However, there are limits to the application of Koch's Postulates which include [1] the inability of certain microorganisms to grow *in vitro* [2] the presence of some microorganisms in healthy and diseased individuals (microorganisms in a carrier state) and [3] the fact that some microorganisms only cause disease under certain environmental conditions (Fredricks and Relman, 1996).

1.4.1 Transmission studies

The transmission of streptococcosis in fish has been investigated through the use of experimental *in vivo* fish challenges. Researchers have used several different pathogen delivery methods in an attempt to cause an experimental bacterial infection to determine the possible modes of entry such pathogens employ.

***Cohabitation*:** Nile tilapia have been successfully infected with *S. agalactiae* through cohabitation with diseased fish (ratio 5:2) (Mian *et al*., 2009). After introduction, the initial healthy fish showed clinical signs of disease 24 – 72 hours later and by day 10 there was 100% mortality. Disease caused by *S. agalactiae* was also shown to be transmissible through cohabitation using golden shiners (*N. crysoleucas*).

Perera *et al*. (1997) were not able to infect tilapia (*O. aureus*) with *S. iniae* through cohabitation. Introduction of healthy fish to diseased fish (ratio 10:5) did not cause the healthy fish to become diseased within the 21 day experiment. However, Shoemaker *et al*. (2000) were able to induce mortality in Nile tilapia through cohabitation. Five moribund or dead *S. iniae* infected tilapia were exposed to 100 healthy fish for 48 hours before their removal from the tank. After 28 days there was 24% total mortality. During this time, researchers noted cannibalism of the eyes and viscera which is a frequent phenomenon in fish and very common in young Nile tilapia (Abdel-Tawwab *et al*., 2006; Fessehaye *et al*., 2006). This incorporates another mode of infection, an oral and/or olfactory mode (Shoemakers *et al*., 2000), which may be responsible for the mortalities that ensued during the cohabitation challenge or was a contributing factor.

***Bath immersion*:** The ability to cause *S. agalactiae* infection through bath immersion has had varied success. Species such as golden shiners (Robinson and Meyer, 1966) and Nile tilapia (Mian *et al*., 2009; Rodkhum *et al*., 2011) that were immersed in a bacterial suspension resulted in mortality and clinical signs of disease. Whereas, red tilapia (*Oreochromis* sp.) did produce some clinical signs of disease when immersed in 3×10^5 cfu/ml for 30 minutes but no fish mortality occurred and fish appeared to recover from infection after 6 days (Abuseliana *et al*., 2010). Furthermore, Rasheed and Plumb (1984) carried out several water-borne challenges by exposing gulf killifish (*Fundulus grandis*) to *S. agalactiae* suspensions of 4×10^{10} cfu/ml for various time periods and under various environmental conditions designed to stress the fish. It was found that, irrespective of the time of exposure, fish showed no sign of infection under a dip treatment when no stress settings were utilised. However, results indicated that the infective ability of this pathogen vastly increased when fish were subjected to epidermal scarification prior to the oral administration of the bacterial suspension (Rasheed and Plumb, 1984).

Chang and Plumb (1996) also stated that injury to the epithelium is a major contributing factor to disease susceptibility of Nile tilapia with *S. iniae*. However, Chang and

Plumb (1996) did not appear to use any 'non-injured' fish to act as a control. Furthermore, Bromage and Owens (2002) demonstrated that epidermal scarification was not necessarily required to cause *S. iniae* infection during a bath immersion for barramundi. Tilapia (*O. niloticus* and *O. niloticus* x *O. aureus*), barramundi (*Lates calcarifer*) and Japanese flounder (*Paralichthys olivaceus*) have all shown to be successfully infected with *S. iniae* through bath immersion (Bromage and Owens, 2002; Nguyen *et al*., 2002; Perera *et al*., 1997; Shoemaker *et al*., 2000).

***Intraperitoneal injection*:** Intraperitoneal injection is the most common method used during experimental bacterial challenges. This transmission route has been successful in *S. agalactiae* (Abuseliana *et al*., 2010; Wongsathein, 2012) and *S. iniae* (Bromage and Owens, 2002; Perera *et al*., 1997) challenge models.

Gills and nares inoculation: Evans *et al*. (2000) conducted an experimental, bilateral inoculation of *S. iniae* onto the eyes or into the nares of hybrid striped bass (*Morone chrysops* x *M. saxatilis*) and tilapia (*O. niloticus*). Although inoculation of the eyes did not result in mortality or disease signs in either species, they were observed following nares inoculation (Evans *et al*., 2000). A study by McNulty *et al*. (2003) further indicated that *S. iniae* could also enter hybrid striped bass though the gills. The subsequent infection was found to have disseminated into the intestinal tract thus supporting the theory that *S. iniae* could be released into the water through faecal matter.

Oral administration: Additional oral routes of *Streptococcus* sp. infection have been proposed due to the ingestion of contaminated diets (Minami, 1979), through peroral inoculation with food (Taniguichi, 1982a; Taniguichi, 1982b) or through cannibalism of dead and/or moribund fish (Shoemaker *et al*., 2000).

There are several different methods of orally administrating bacteria into a susceptible host in an experimental setting. Rasheed and Plumb (1984) used polyethylene tubing to directly dispense *S. agalactiae* into the stomach of Gulf killifish. This did not cause any fish mortality, however. A plastic catheter was also used by Perera *et al*. (1997) to administer *S. iniae* into the gut of tilapia, which successfully caused infection and mortality. Similarly, Japanese flounders became diseased when *S. iniae,* in a pellet feed/bacterial suspension slurry, was intragastrically injected using a plastic catheter. Bromage and Owens (2002) created fish food pellets infected with *S. iniae* which, once fed to barramundi, led to infection and mortality.

The vertical transmission of *S. agalactiae* from parent to offspring was previously thought not to occur (Hernández *et al*., 2009; Jiménez *et al*., 2011). However, recent work from Suebsing *et al*. (2013) suggests that both *S. agalactiae* and *S. iniae* could be vertically transmitted. Under experimental conditions the modes of *Streptococcus* sp. transmission are numerous and include horizontal, oral and water-borne routes. However, as stated by Zlotkin *et al*. (1998), since cultured fish exist in an environment where food, faeces and water is undividable, it is clear that infection is also spread from the immediate surroundings. Furthermore, organic matter, mud, and even seawater may act as a reservoir for pathogenic organisms (Zlotkin *et al*., 1998). From an experimental perspective it is important to note that the fish species, bacterial species and bacterial isolates being investigated may affect the route of transmission into a susceptible host and thus explain differences found within the literature.

1.4.2 Virulence factors of *Streptococcus agalactiae* and *Streptococcus iniae*

Koch's postulates were reformed by Falkow (1988) to incorporate microbial genetic and molecular cloning. The criteria in the subsequently named 'molecular Koch's postulates' is often used to aid the depiction of virulence factors and include: [1] The phenotype or property under investigation should be associated with pathogenic strains. [2] Specific inactivation of the gene(s) associated with the suspected virulence trait should lead to a measurable loss in pathogenicity/virulence. [3] Reversion/allelic replacement of the mutated gene should lead to restoration of pathogenicity. Pathogenicity is a qualitative trait that refers to the ability of an organism to cause disease in a host organism, whereas virulence is a quantitative trait representing the severity of the pathology caused by the pathogen. Virulence factors encompass an array of bacterial products or strategies that contribute to virulence or pathogenicity (Segura and Gottschalk, 2004).

The pathogenesis of *S. iniae* infection is a multistep process (Zlotkin *et al*., 2003). The instigation of this disease is believed to occur through the colonisation of external tissue, followed by local spread and subsequently invasion of the bloodstream (Zlotkin *et al*., 2003). Once in the bloodstream bacteria are thought to inhabit the central nervous system (CNS) of its host by passing through the blood-brain barrier as free bacteria or be carried in associated with monocytes or phagocytes (Agnew and Barnes, 2007; Zlotkin *et al*., 2003). The latter process is described as the 'Trojan horse effect'. *Streptococcus iniae* loaded within macrophages are able to withstand macrophage bactericidal activities and can trigger apoptosis to facilitate their release around the host's body (Agnew and Barnes, 2007; Zlotkin *et al*., 2003). Such a process is also thought to prevent the triggering of the host's defence mechanisms. However, work from Locke *et al.* (2007) may contradict such a theory as it was found that strains covered by a polysaccharide capsule are more virulent in fish than their non-encapsulated counterparts. Such results indicate that phagocyte colonisation and survival may not be the principlal infection strategy utilised by *S. iniae* (Agnes and Barnes, 2007) otherwise there would be little need for the bacterium to have opsonophagocytosis resistance mechanisms.

Less is known about the pathogenesis of *S. agalactiae* infection in fish. However this bacterium is also thought to utilise macrophages to cross the blood-brain barrier and disseminate in organs and tissues (Bowater *et al*., 2012). Similar to *S. iniae*, *S. agalactiae* may induce apoptosis or necrosis in macrophages but it is hypothesised that haemolysin may contribute to this mechanism of phagocyte killing (Guo *et al*., 2014). The target organs of *S. agalactiae* are the brain, eye and kidney, all similar to *S. iniae*, and vasculitis and septicaemia are the major pathogenic effects of *S. agalactiae* (Abdullah *et al*., 2013).

The pathogenesis of *S. agalactiae* and *S. iniae* is not yet fully understood as it is a complex and multifactorial process. Since pathogenesis is attained through the bacterium's virulence factors, many researchers have focused upon identifying and characterising these. Both bacterial species produce and secrete a variety of products that contribute to adherence, colonisation, invasion and protective immunity. The available information regarding virulence factors of *S. agalactiae* and *S. iniae* in the literature research are shown in Tables 1.7 and 1.8 and their regulation depicted in Figures 1.4 and 1.5, respectively. Virulence factors of *S. agalactiae* are commonly examined using isolates that were obtained from mammalian hosts and tested in mammalian models; the only exception to this is work from Guo *et al*. (2014) (not included in the table) where an *S. agalactiae* isolate from a tilapia was used but a mammalian cell line was employed. It cannot be assumed that the findings presented in Table 1.7 are directly applicable to aquatic strains. However, Delannoy *et al*. (2013) identified 4 subpopulations of aquatic *S. agalactiae* strains and, of these 4 subpopulations, 3 were also found in human isolates. Using aquatic *S. agalactiae* strains some researchers have screened for virulence genes (Delannoy *et al*., 2013; Godoy *et al*., 2013) however, the mere presence of such genes does not signify expression and therefore an inaccurate illustration of the bacterium's pathogenesis may be conveyed.

Table 1.7 Known virulence factors of *Streptococcus agalactiae.* All *S. agalactiae* strains were isolated from mammals and the virulence factors were verified using mammalian based models.

Virulence factor	Related genes	Function	Reference
Fibrinogen-binding protein	*fbs*	A fibrinogen-binding protein that contributes to adhesion to host surfaces, protects from opsonophagocytosis and elicits a fibrinogen-dependent aggregation of platelets.	Pietrocola *et al*., 2005; Schubert *et al*., 2002
Laminin-binding protein (Lmb)	*lmb*	Laminin-binding lipoprotein that mediates attachment to laminin which may play a critical role in bacterial colonization.	Spellerberg *et al*., 1999
C5a peptidase	*scpB*	A surface-associated serine protease which leaves C5a, a major neutrophil chemoattractant, and facilitates adherence to fibronectin.	Bechmann *et al*., 2002
Hyaluronate lyase	*hylB*	Degrades hyaluronan, the main polysaccharide component of the host connective tissues and facilitates bacterial invasion.	Mello *et al*., 2002
B-haemolysin/cytolysin	cyl	A surface-associated toxin with the ability to promote intracellular invasion and neutrophil recruitment, trigger apoptosis of cells and cause cytolytic injury.	Liu and Nizet, 2006
Cβ protein	*bac*	IgA binding protein important in bacterial resistance to mucosal immune defence mechanisms.	Kong *et al*., 2006
Resistance to protease immunity protein	*rib*	Cell surface protein that confers protective immunity.	Stålhammar-Carlemalm *et al*., 1993
Cα protein	*bca*	Surface protein that plays a role in the interaction with epithelial surfaces and initiation of infection.	Bolduc *et al*., 2002; Li *et al*., 1997
CAMP factor	*cfb*	Pore-forming toxin that causes lysis of red blood cells and binds to the Fc fragments of immunoglobulin.	Lang and Palmer, 2003
Capsule	*cps*	Polysaccharide capsule that reduced complement deposition and phagocytosis by the host's immune systems.	Hanson *et al.,* 2012; Yamamoto *et al*., 1999
Surface protein of group B	*spb*	Mediates internalization by contributing towards epithelial cell adherence and invasion.	Adderson *et al*., 2003
Superoxide dismuase	*sodA*	Enzyme that provides protection from oxidative stress and contributes towards survival in macrophages.	Poyart *et al*., 2001
Glyceraldehyde 3-phosphate dehydrogenases (GAPDH)	*gapC*	Glycolytic enzyme involved in bacterial energy generation, plays a role in adhesion to host components and can induce apoptosis in host macrophages.	Madureira *et al*., 2007; Oliveria *et al*., 2012;
Immunogenic adhesion (bibA)	*bibA*	Surface protein that has a role in adhesion and resistance to opsonophagocytic killing by host's neutrophils.	Santi *et al*., 2007
Hyaluronidase	*hylB*	An enzyme that contributes to virulence by breaking down the substrate hyaluronic acid, a component of the extracellular matrix in some tissues.	Sukhnanand *et al*., 2005
Serine proteinase	*cspA*	Protease that inactive chemokines and aids the capacity to resist opsonophagocytic killing by neutrophils.	Bryan and Shelver, 2009
Invasion-associated gene glycosyltransferase	*iagA*	The glycolipid product of *iagA*, diglucosyldiacylglycerol, is a cell membrane anchor for lipoteichoic acid and plays a role in penetrating the blood-brain barrier.	Doran *et al*., 2005
Pili	PI-1, PI-2a and	Mediates resistance to cathelicidin antimicrobial peptides.	Maisey *et al*., 2008; Papasergi *et al*., 2011

	PI-2b		
Serine-rich repeat proteins	*srr*	Contributes to the host cell attachment by binding to keratin on the surface of host cells.	Sheen *et al*., 2011
Methionine transport regulator	*mtaR*	Essential for normal growth in plasma and normal methionine transport.	Shelver *et al*., 2003
D-alanylation of lipoteichoic acid	*dlt*	Influences the surface charge on the cell surface, reduces susceptibilty to cationic antimicrobial peptides and killing my phagocytes.	Poyart *et al*., 2003

Table 1.8 Known virulence factors of *Streptococcus iniae*

Virulence factor	Related genes	*S. iniae* strains isolated from	Model used to verify virulence factor	Function	Reference
Interleukin-8 protease (IL-8)	*cepI*	Fish and mammal	Mammal	Protein that acts as a potent chemoattractant, prominent role in recruitment and activation of neutrophils.	Zinkernagel *et al*., 2008
Streptolysin S (SLS)	*sagA*	Fish and mammal	Mammal	Pore-forming cytotoxin that promotes local tissue necrosis.	Fuller *et al*., 2002
CAMP factor	*cfi*	Mammal	Mammal	Pore-forming toxin with cytolytic activity and the ability to bind immunoglobulin.	Bolotin *et al.,* 2007
Transcriptional regulator CpsY	*cpsY*	Mammal	Fish and mammal	Required for intracellular survival in neutrophils.	Allen and Neely, 2011
α-enolase	*Not known*	Fish	Mammal	Protein that contributes to the ability of *S. iniae* to cross tissue barriers through plasminogen activation.	Kim *et al*., 2007
C5a peptidase	*scpI*	Fish	Fish	Surface protein that impairs the ability of the host to fight an *S. iniae* infection.	Locke *et al*., 2008
Capsule	*cpsD*	Fish	Fish	Surface capsular polysaccharide that lowers the rate of phagocytosis by host immune cells.	Locke *et al*., 2007
Extracellular polysaccharide (EPS)	*Not known*	Fish	Fish	Triggers proinflammatory cytokines.	Eyngor *et al*., 2010
Phosphoglucomutase gene	*pgm*	Fish	Fish	Contributes to normal cell morphology, surface capsule expression and resistance to innate immune clearance mechanisms.	Buchanan *et al*., 2005
Polysaccaride deacetylase	*pdi*	Fish	Fish	Virulence proteins involved in adherence and invasion, lysozyme resistance and survival in blood.	Milani *et al*., 2010
SiM protein	*simA/ simB*	Fish	Fish	Contributes to bacterial adherence, invasion of fish epithelial cells and macrophage resistance.	Locke *et al*., 2008
Streptolysin S	*sagA*	Fish	Fish	Expression contributes directly to cytolytic injury to cells and tissues.	Locke *et al*., 2007

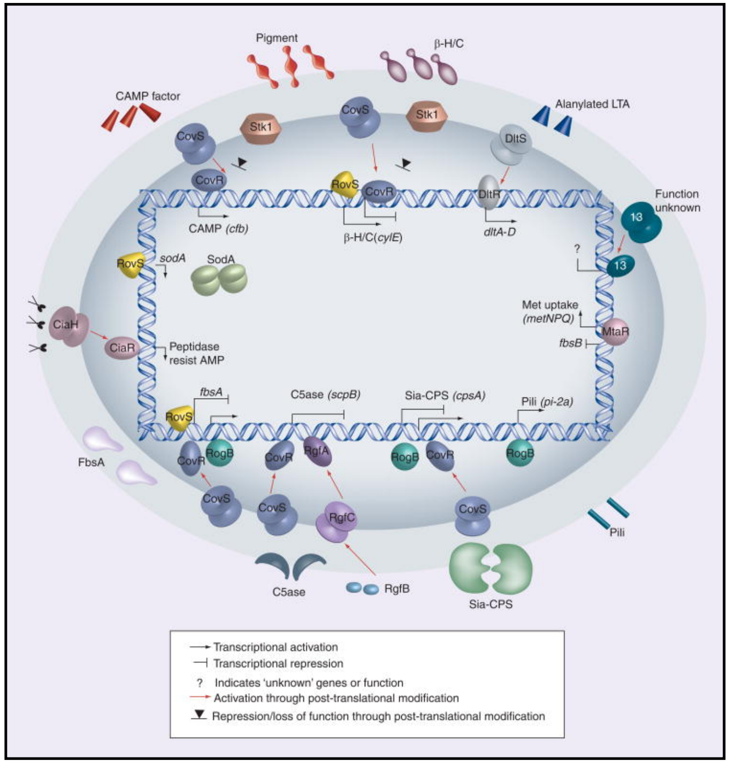


Figure 1.4 Regulation of factors important for Group B streptococcal disease pathogenesis. Two-component system (TCS) comprising the response regulators CovR, RgfA, CiaR and DltR, and their cognate sensor histidine kinases CovS, RgfC, CiaH and DltS, regulate the transcription of toxins and other factors that contribute to GBS virulence. β-H/C: β-hemolysin/cytolysin; AMP: Antimicrobial peptide; C5ase: C5a peptidase; CAMP: Christie Atkins Munch Peterson; FbsA: Fibrinogen-binding protein A; GBS: Group B Streptococci LTA: Lipotechoic acid; Met: Methionine; Sia-CPS: Sialic acid capsular polysaccharide; SodA: Superoxide dismutase. Taken from Rajagopal (2009).

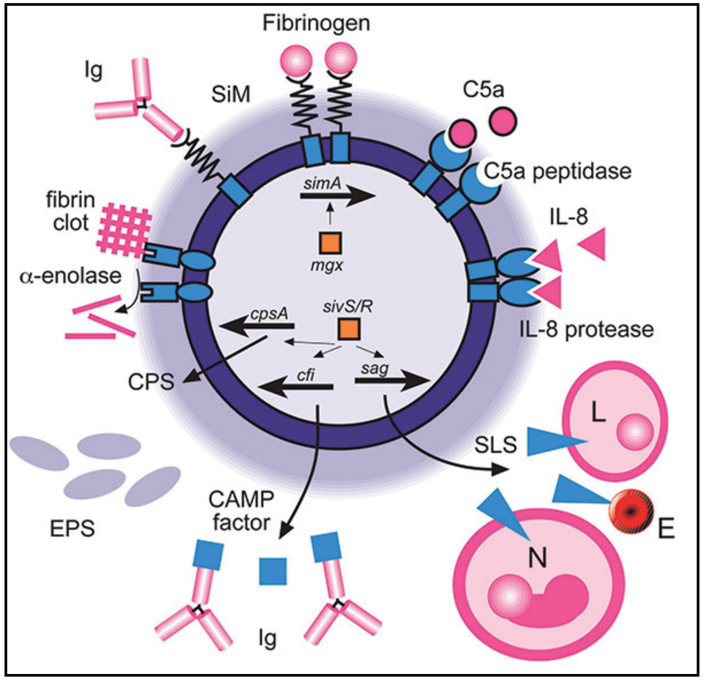


Figure 1.5 Virulence factors of *Streptococcus iniae*. SiM protein (simA) expression is likely to be regulated by mgx, SiM protein binds immunoglobulin (Ig) and fibrinogen. C5a peptidase and interleukin-8 (IL-8) protease degrade their respective chemokines to impair phagocyte signaling. Production of the cytolysin streptolysin S (sag; SLS) is regulated by the sivS/R system. SLS lyses lymphocytes (L), erythrocytes (E), and neutrophils (N). The CAMP factor gene, cfi, is also regulated by sicS/R and is known to bind immunoglobulin by the Fc region. Capsular polysaccharide (cpsA; CPS) synthesis is controlled by sivS/R and is represented by a haze around the cell. Exopolysaccharide (EPS) is produced in excess and contributes to highly viscous growth. α–enolase degrades fibrin clots and promotes dissemination. Taken from Baiano and Barnes (2009).

1.5 Aim of study

Host-pathogen interactions seldom occur on a one-to-one basis yet aquaculture research primarily focuses upon a single disease agent (Xu *et al*., 2012). Simultaneous infections, alternatively called concurrent or co-infections, are just starting to gain attention within aquaculture research. Resultantly, to date there are only a limited number of co-infection studies that have been conducted and the majority of these are based on parasite-bacteria interactions within a fish. However, there are a few investigations that have studied co-infections with two different bacterial species such as that from Crumlish *et al.* (2010).

Streptococcosis has been described by Conroy (2009) as evolving from an 'emerging pathology into a true, fully identified, well-established entity'. *Streptococcus agalactiae* and *S. iniae* are known to both exist in several countries and it has been found that an epizootic outbreak caused by one these pathogens can be followed by another outbreak from the second bacterium present (Conroy, 2009). It can therefore be assumed that these pathogens may actually be on the same fish farms at the same time. The repercussions these two pathogens are having on the tilapia aquaculture industry are vast yet there is no information regarding *S. agalactiae* and *S. iniae* co-infections. Such information would be valuable for understanding the epidemiology and for the treatment and control of streptococcosis.

Concurrent infections are complex in natural conditions as well as under experimental studies and subsequently involve a substantial amount of research to provide sufficient data for applicable conclusions to be drawn. Consequently, this study intended to provide a solid foundation upon which future work could expand by investigating the pathogenesis of aquatic *S. agalactiae* and *S. iniae* in tilapia (*O. niloticus*). The objectives of the study were:

[1] To evaluate and determine the most useful techniques for the detection and identification of these two pathogens.

[2] To identify the expression of virulence factors *in vitro* for *S. agalactiae* and *S. iniae* and compare any inter and intra-species variation.

[3] To assess if there is competition or coexistence between *S. agalactiae* and *S. iniae in vitro.*

[4] To develop a robust and reliable challenge model for *S. agalactiae* and *S. iniae* in Nile tilapia using intraperitoneal injection.

[5] To perform a sequential challenge for [1] *S. agalactiae* [2] *S. iniae* and [3] *S. agalactiae* and *S. iniae* in Nile tilapia.

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Chapter 2

Bacterial identification and characterisation of aquatic *Streptococcus agalactiae* and *Streptococcus iniae* isolates

2.1 Introduction

Streptococcus agalactiae and *S. iniae* are two bacterial pathogens affecting cultured and wild populations of fresh and marine fish species throughout the world (Agnew and Barnes, 2007; Mian *et al.*, 2009). Clinical signs of disease due to these organisms can vary between the host species affected, however they are generally similar to those of other streptococcal infections (Agnew and Barnes, 2007; Mata *et al.*, 2004). Although the treatment and management strategies implemented in the event of a streptococcal disease outbreak are comparable (Section 1.3.7), an accurate diagnosis is essential and the pathogen causing the disease needs to be established. This information is vital for monitoring the spread of a disease outbreak and for the development of preventative controls such as vaccines.

Existing protocols and techniques used for the identification of *Streptococcus* spp. include: agar plate cultivation, conventional biochemical and phenotypic assays, Lancefield serogrouping, enzymatic profiles, whole cell fatty acid analysis, indirect fluorescent antibody technique (IFAT) (Klesius *et al.*, 2006) and species-specific polymerase chain reactions (PCR). However, *Streptococcus* spp. have relatively similar phenotypic profiles and there is also intra-species variation, which complicates identification proceedings. Furthermore, not all of the identification methods will be available or applicable for each individual case or in each diagnostic facility.

The aim of this study was to determine the minimal identification tests required to differentiate between *S. agalactiae* and *S. iniae* when recovered from a streptococcal infection in fish. This was performed using a range of isolates obtained from various disease outbreaks across a wide geographical range. These assays would then be used in subsequent experimental studies to confirm pathogen identification.

2.2 Materials and Methods

2.2.1 Bacterial strains and growth conditions

Streptococcus agalactiae, S. iniae and other bacterial species were obtained from cryo-bead (Technical Services Consultants Ltd, Heywood, UK) culture collections stored at -70 °C at the Institute of Aquaculture, University of Stirling. The strains used in this study included type strains from commercial culture collections and reference isolates from clinical fish disease cases. The source of the isolation and/or the geographical origin of the bacterial strains used in this study are presented in Table 2.1.

To revive the bacteria from storage an individual bead was inoculated onto tryptone soya agar (TSA) (Oxoid Ltd, Basingstoke, UK) then incubated at 28 °C for 48 hours. The isolates were identified using phenotypic characterisation and PCRs. For DNA extraction, a single colony from each bacterial strain was inoculated into 5 ml tryptone soya broth (TSB) (Oxoid Ltd, Basingstoke, UK) and incubated at 28 °C for 24 hours with shaking at 140 rpm (Kühner Shaker ISF-1-W, Adolf Kühner AG, Switzerland).

Table 2.1 Bacterial species and strains used in this study

Bacterial species	Strain identification	Country	Year	Host
Streptococcus agalactiae	09011056/1L	Thailand	2009	Tilapia (*Oreochomis niloticus*)
	09011088/2L	Thailand	2009	Chinese bullfrog (*Rana rugulosa*)
	B08059 28E	Honduras	2008	Tilapia (*Oreochomis niloticus*)
	B08065 42H	Honduras	2008	Tilapia (*Oreochomis niloticus*)
	B09032 Sa Ti Be 08 – 18 b	Colombia	2008	Tilapia (*Oreochomis niloticus*)
	B09032 Sa Ti Be 08 – 21 a	Colombia	2008	Tilapia (*Oreochomis niloticus*)
	B09032 Sa Ti Be 08 – 21 b	Colombia	2008	Tilapia (*Oreochomis niloticus*)
	B09032 Sa Ti Be 08 – 18 a	Colombia	2008	Tilapia (*Oreochomis niloticus*)
	B09032 Sa Ti Cr 08 – 14 b	Costa Rica	2008	Tilapia (*Oreochomis niloticus*)
	K0101	Kuwait	2001	Mullet (*Liza klunzingeri*)
	K0102	Kuwait	2001	Mullet (*Liza klunzingeri*)
	K0103	Kuwait	2001	Mullet (*Liza klunzingeri*)
	K0104	Kuwait	2001	Mullet (*Liza klunzingeri*)
	K0105	Kuwait	2001	Mullet (*Liza klunzingeri*)
	May 06 – 6	Vietnam	2006	Tilapia (*Oreochomis niloticus*)
	Vitafish 01	Belgium	2007	Tilapia (*Oreochomis niloticus*)
	Vitafish 02	Belgium	2007	Tilapia (*Oreochomis niloticus*)
Streptococcus iniae	B08065 50H	Honduras	2008	Tilapia (*Oreochomis niloticus*)
	B99115	Barbados	1999	n/a
	B99120 1090/99	Barbados	1999	Parrot fish (*Sparisoma aurofrenatum)*
	B99120 1103/99	Barbados	1999	Snapper (*Ocyurus chrysurus*)
	B99120 1104/99	Barbados	1999	Grunt (*Haemulidae* sp.)
	B99120 1105/99	Barbados	1999	Grunt (*Haemulidae* sp.)
	B99120 1121/99	Barbados	1999	Chubb (*Scaridae* sp.)
	B99120 1121/99 P	Barbados	1999	Parrot fish (*Sparisoma viridae*)
	B99130 B	Grenadines	1999	Reef fish
	B99130 K	Grenadines	1999	Reef fish
	J39	Korea	2001	Olive flounder (*Paralichthys olivaceus*)
Aeromonas hydrophila	NCIMB 9240	n/a	n/a	n/a
Bacillus subtilis	NCIMB 3610	n/a	n/a	n/a
Enterobacter aerogenes	NCIMB 10102	n/a	n/a	n/a
Enterobacter cloacae	NCIMB 10101	n/a	n/a	n/a
Enterococcus faecalis	NCIMB 775	n/a	n/a	n/a
Lactococcus garvieae	NCIMB 702155	n/a	n/a	n/a
Streptococcus agalactiae	NCIMB 701348	n/a	n/a	n/a
Streptococcus iniae	ATCC 29178	n/a	n/a	n/a
Escherichia coli [1]	ATCC 25922	n/a	n/a	n/a
Escherichia coli [2]	NCIMB 86	n/a	n/a	n/a

[ATCC] American Type Culture Collection [NCIMB] The National Collection of Industrial Food and Marine Bacteria [n/a] Not available/Not applicable

2.2.2 Phenotypic characterisation of the bacterial isolates

Type strains of *S. agalactiae* National Collection of Industrial, Food and Marine Bacteria (NCIMB) 701348 and *S. iniae* American Type Culture Collection (ATCC) 29178 were used as positive controls and for validation of all assays.

Once bacteria were revived from cryo-bead cultures, colony morphology was observed on the TSA plates and cell morphology was assessed with Gram-stained smears as described in Frerichs and Millar (1993) (See appendix). The selective agar, Edwards medium (modified) (Oxoid Ltd, Basingstoke, UK) was used for the initial determination of *Streptococcus* species. Subcultures of pure bacterial growth from TSA plates were subsequently grown on Edwards medium for 48 hours at 28 °C; positive results were indicated by the formation of blue coloured colonies. *Enterococcus faecalis* was used as a positive control and *Lactococcus garvieae* and *Escherichia coli* [1] (Table 2.1) were used as negative controls.

The oxidase test (Sigma-Aldrich, Buchs, Switzerland) was used to detect the cytochrome oxidase enzyme. The beta-haemolytic activity was assessed by growing individual bacterial colonies for 24 – 72 at 28 °C hours on sheep blood agar (SBA) [blood agar base (Oxoid Ltd, Basingstoke, UK) with 5% (v/w) sheep red blood cells].

Isolates were characterised biochemically with the API 20 STREP test and serologically with Slidex Strepto-kit (both from biomérieux, Marcy l'Etoile, France). These tests were performed according to the manufacturer's instructions with the exception that API 20 STREP strips were incubated at 28 °C instead of the recommended 36 °C.

2.2.3 Temperature and salt tolerance assays

From a pure culture, colonies of each bacterial isolate were transferred into 2 ml sterile distilled water with the bacterial density adjusted to a MacFarland standard of 0.5 [bacterial density ≈ 1.5 x 10^8/ml]. A 100 µl sample of each bacterial suspension was then added to 5 ml of TSB. To determine temperature tolerance, each isolate was incubated at 4, 15, 22, 28, 37, 43 and 47 °C alongside the negative controls which consisted of TSB with no bacteria.

Salinity tolerance was investigated by growing isolates at 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 6.5 and 7.0% (w/v) sodium chloride (NaCl) in TSB at 28 °C. The bacterial suspensions were prepared as described above. Negative controls comprising of TSB with no bacteria were made for each salinity group. For both assays viable growth was compared with the controls over 4 days.

2.2.4 Biochemical assays and profiling of bacterial isolates

2.2.4.1 Differentiation between *Streptococcus agalactiae* and *Streptococcus iniae*

The starch hydrolysis test was based on the protocol in Cowan and Steel (2003). Briefly, TSA containing 0.2% soluble starch (BDH laboratory supplies, Poole, England) was inoculated with bacteria and incubated for 48 hours at 28 °C. After this time the agar plates were flooded with Lugol's iodine solution and checked for the formation of clear colourless zones indicating a positive starch hydrolysis result. *Bacillus subtilis* and *E. coli* [2] (Table 2.1) were used as positive and negative controls respectively.

2.2.4.2 Comparisons between commercial tests and conventional techniques

Using conventional techniques a short validation study was conducted looking into the results obtained using an API 20 STREP. The conventional biochemical tests selected included reactions that showed distinct differences between *S. iniae* and *S. agalactiae* isolates in an API 20 STREP reaction [Voges-Proskauer (VP), amygdalin and aesculin]. Other tests that were chosen included assays that were negative for all *S. agalactiae* and *S. iniae* isolates (sorbitol) or expressed variable results between bacterial strains of the same species [arginine dihydrolase (ADH)].

Voges-Proskauer

Colonies of each bacterial isolate taken from a pure culture were transferred into 2 ml sterile distilled water with the bacterial density adjusted to a MacFarland standard of 2 [bacterial density ≈ 6 x 10^8/ml]. The VP reaction was based on the O'Meara's method (1931) (cited by Cowan and Steel, 2003). Briefly, a 100 µl sample of each bacterial suspension was added to 5 ml Methyl-Red and Voges-Proskauer broth (MR-VP) (Oxoid Ltd, Basingstoke, UK) and incubated at 28 °C. Following an incubation period of 48 hours, 50 µl of creatin solution (1% creatin monohydrate (Sigma-Aldrich, Buchs, Switzerland) in 0.1 M HCl) and 1 ml of 40% potassium hydroxide solution was added, results were taken after 1 and 4 hours. Positive results were indicated by an eosin-pink colour and the test recorded as negative if no colour change was observed. *Enterobacter aerogenes* was used as a positive control.

Arginine dihydrolase

The test for arginine dihydrolase was based on the method developed by Falkow (1958). Colonies of each bacterial isolate taken from a pure culture were transferred into 2 ml sterile distilled water with the bacterial density adjusted to a MacFarland standard of 2. A 100 µl sample of each bacterial suspension was added to 5 ml of decarboxylase medium readjusted to a pH 6.7 (control tube). This was repeated with decarboxylase medium (Becton, Dickinson and Company, Le Pont de Claix, France) containing 0.5% arginine hydrochloride (Sigma-Aldrich, Buchs, Switzerland) (ADH tube). All samples were placed in anaerobic conditions through the addition of sterile liquid paraffin oil and incubated at 28 °C. Colour changes were observed at 24-hour internals for a period of 4 days. A yellow colour in the control tube and a violet colour in the ADH tube indicated a positive result. A yellow colour in both the control and ADH tube indicated a negative result. *Enterobacter cloacae* was used as a positive control.

Sorbitol and amygdalin hydrate

The method for assessing carbohydrate profile reactions was adapted from Waltman *et al.* (1986). One percent sorbitol or amygdalin hydrate (Alfa Aesar, Heysham, UK) was added to oxidation/fermentation (OF) basal medium (Becton, Dickinson and Company, Le Pont de Claix, France) containing 0.1% Agar No. 1 (Oxoid Ltd, Basingstoke, UK). The medium was adjusted to pH 6.8 before 9 ml was transferred to an appropriate container. A single colony of each bacterium was added to the hardened media and placed in aerobic conditions (open tube); this was repeated with the addition of sterile liquid paraffin oil to create an anaerobic environment (closed tube). Samples were incubated at 28 °C with any colour changes observed at 24 hour intervals for a period of 7 days. A green colour in both the open and closed tubes indicated a negative result, a yellow colour in both tubes indicated a positive fermentative result and a yellow colour in the open tube and a green colour in the closed tube indicated a positive oxidative result.

Aesculin

To test the ability of *S. agalactiae* and *S. iniae* isolates to hydrolyse aesculin two different methods were used. [1] Bacteria were aseptically streaked onto bile aesculin agar (Oxoid Ltd, Basingstoke, UK) as described by Cowan and Steel (2003) and [2] a bacterial suspension was spread onto TSA with a bile-aesculin disk (Sigma-Aldrich, Buchs, Switzerland). When using a bile-aesculin disk, colonies of each bacterial isolate taken from a pure culture were transferred to 2 ml sterile distilled water with the bacterial density adjusted to a MacFarland standard of 2. A 50 µl sample of each bacterial suspension was then spread onto TSA plates to produce a bacterial lawn before a bile-aesculin disk was placed centrally onto the agar. Plates from both tests were incubated for 48 hours at 28 °C and then observed for blackening of the medium which indicated a positive reaction. *Aeromonas hydrophila* was used as a positive control for both tests.

2.2.5 DNA extraction

DNA was extracted from each bacterial isolate following a modified version of the Seward *et al.* (1997) method. Bacteria were grown as described previously (Sections 2.2.1) then harvested by centrifugation at 2602 g for 15 minutes at 4 °C (MSE Mistral 2000R, MSE, London, UK). The sample supernatant was discarded and the cell pellet resuspended in 1.0 ml of Sodium Chloride-Tris-Ethylenediaminete-traacetic acid (STE) buffer (See appendix) and then centrifuged for 1 minute at 12470 g (Sigma 1-14 Microfuge, Sigma, Osterode am Harz, Germany). The supernatant was again discarded prior to the cell pellet being resuspended in 100 µl of Tris-EDTA (TE) buffer (See appendix) and then boiled at 95 °C for 10 minutes. A final centrifugation was performed (1 minute at 12470 g) and the upper aqueous phase containing the sample's DNA was removed. The concentration (ng/µl) and quality (260/280 ratio) of the DNA extractions were measured by a spectrophotometer (Nanodrop ND1000, Thermo Fisher Scientific Inc, Wilmington, USA). The DNA samples were stored in sterile tubes in 20 µl aliquots at -20 °C until required.

2.2.6 Polymerase chain reaction (PCR) and gel electrophoresis

Appropriate controls were included within the PCR protocol; a positive control for DNA and primers (recognised species from bacterial culture collections), a negative control for PCR mix (Milli-Q water with no DNA) and controls to illustrate primer specificity. These included the type strains of *S. iniae*, *S. agalactiae*, *E. faecalis* and *L. garvieae*. The oligonucleotide primers used for the detection of *S. agalactiae* or *S. iniae* isolates with a DNA gel electrophoresis are shown in Table 2.2.

Amplification of each DNA sample was performed in a 25 µl reaction mixture using a master mix that contained 2.5 µl 10 x reaction buffer, 2 µl $MgCl_2$ (25 mM), 0.5 µl Klear Taq (5 U/µl) (all from Kbioscience, Massachusetts, USA), 0.5 µl dNTP (20 mM) (Thermo Fisher Scientific, Surrey, UK), 1.5 µl of each oligonucleotide primer (Eurofins MWG Operon, Germany), approximately 1000 ng/ µl of template DNA, and Milli-Q water to volume. The PCR was carried out in a Biometra thermal cycler (Biometra, Goettingen, Germany).

The PCR parameters used for primer sets Sin-1 – Sin-2, 5'144 – 3'516, LOX-1 – LOX-2, F1 – IMOD and STRA-AgI – STRA-AgII were an initial denaturation at 95 °C for 15 minutes, 35 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 25 seconds, with a final extension for 10 minutes at 72 °C. For the SP-1 – SP-2 primer set the recommended optimised PCR parameters were used (Zhou *et al.*, 2011): an initial denaturation at 95 °C for 15 minutes, 35 cycles of 94 °C for 1 minute, 60 °C for 1 minute and 72 °C for 1 minute, with a final extension for 10 minutes at 72 °C. All amplified products were stored at -20 °C until used.

Table 2.2 Oligonucleotide primers for the identification of *Streptococcus agalactiae* or *Streptococcus iniae*

Primer	Direction	Nucleotide sequence (5' – 3')	Target gene	Target region (bp)	Pathogen	Reference
Sin-1	Forward	CTAGAGTACACATGTAGCTAAG	16S rRNA	300	*S .iniae*	Zlotkin *et al.*, 1998
Sin-2	Reverse	GGATTTTCCACTCCCATTAC				
LOX-1	Forward	AAGGGGAAATCGCAAGTGCC	*lctO*	870	*S. iniae*	Mata *et al.*, 2004
LOX-2	Reverse	ATATCTGATTGGGCCGTCTAA				
5'144	Forward	GGAAAGAGACGCAGTGTCAAAACAC	16S–23S rRNA	373	*S. iniae*	Berridge *et al.*, 1998
3'516	Reverse	CTTACCTTAGCCCCAGTCTAAGGAC				
SP-1	Forward	GAAAATAGGAAAGAGACGCAGTGTC	16S–23S rRNA	377	*S. iniae*	Zhou *et al.*, 2011
SP-2	Reverse	CCTTATTTCCAGTCTTTCGACCTTC				
F1	Forward	GAGTTTGATCATGGCTCAG	16S rRNA	220	*S. agalactiae*	Martinez *et al.*, 2001
IMOD	Reverse	ACCAACATGTGTTAATTACTC				
STRA-AgI	Forward	AAGGAAACCTGCCATTTG	16S–23S rRNA	270	*S. agalactiae*	Phuektes *et al.*, 2001
STRA-AgII	Reverse	TTAACCTAGTTTCTTTAAAACTAGAA				

2.2.6.1 illustra PuReTaq Ready-To-Go PCR Bead

An alternative PCR method using illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Buckinghamshire, UK) was also compared with the method described above. The two different methods were initially trialled on one primer set, STRA-AgI – STRA-AgII, using *S. agalactiae* NCIMB 701348 and *S. agalactiae* 09011088/2L. The amplification conditions for the

Ready-To-Go PCR Bead were as follows: amplification of each DNA sample was performed in a 25 µl reaction mixture containing a single Ready-To-Go PCR Bead, 1.5 µl of each primer and approximately 1000 ng/µl of DNA, and Milli-Q water to volume. The thermal cycle parameters were: initial denaturation at 95 °C for 5 minutes followed by 35 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 25 seconds with a final extension for 10 minutes at 72 °C. All amplified products were stored at -20 °C until used.

2.2.6.2 Duplex-PCR

A duplex-PCR was conducted using both primer sets LOX-1 – LOX-2 and F1 – IMOD (Rodkhum *et al*., 2012) and carried out using both PCR methods previously described. Three DNA samples were used during these reactions: [1] *S. iniae* ATCC 29178 only [2] *S. agalactiae* NCIMB 701348 only and [3] a combined culture mixture of both *S. iniae* ATCC 29178 and *S. agalactiae* NCIMB 701348. The thermal cycle parameters were the same as previously described for each PCR method. All amplified PCR products were stored at -20 °C until used.

2.2.6.3 Visualisation of PCR products

The amplified products were resolved by gel electrophoresis. Analysis was performed on 1.5% (w/v) agarose gels, stained with ethidium bromide, using a Tris-acetate-EDTA (TAE) buffer system (0.5. x Tris-acetate-EDTA) (See appendix). A 10 µl sample of PCR product was mixed with 2 µl of (1x) loading buffer (10X BlueJuice gel loading buffer, Invitrogen, Paisley, UK). A final 10 µl loading volume was used for all samples. Band patterns were visualised and photographed under UV with the size of the restriction fragments estimated by comparison to a 1 Kb DNA ladder with a loading volume of 3 µl (Invitrogen, Paisley, UK). Samples were considered positive when a clear band was observed under UV light at the relevant target region for each primer set.

2.3 Results

2.3.1 Bacterial growth and identification

Following a 48 hour incubation at 28 °C, all isolates developed as small, white colonies on TSA. These were all Gram-positive, oxidase negative cocci, which were commonly observed microscopically in chains. Slidex Strepto-kit tested positive for Group B *Streptococcus* for all *S. agalactiae* isolates except for three strains: *S. agalactiae* B08065 42H, *S. agalactiae* Vitafish 01 and *S. agalactiae* Vitafish 02. All *S. iniae* isolates were negative for this test with the exception of *S. iniae* B08065 50H, which was positive for Group B *Streptococcus*. These four isolates (*S. agalactiae* B08065 42H, Vitafish 01, Vitafish 02 and *S. iniae* B08065 50H) were considered to be mislabelled or misidentified so consequently removed from further analysis in this study.

Enterococcus faecalis and all *S. agalactiae* and *S. iniae* isolates grew on Edwards medium as small, blue colonies after a 48 – 72 hour incubation. *Entercococcus faecalis* also showed fermentation. No growth was observed for the negative control *E. coli* or *L. garvieae.*

Streptococcus iniae isolates were all haemolytic on SBA whereas *S. agalactiae* isolates expressed a higher degree of variability in their haemolytic capability. Nine of the *S. agalactiae* isolates were non-haemolytic, including the type strain *S. agalactiae* NCIMB 701348, and eight isolates expressed weak haemolysis after a 48 – 72 hour incubation.

The salt and temperature tolerance of *S. agalactiae* and *S. iniae* isolates are illustrated in Figure 2.1. All isolates, irrespective of the species, were able to grow at the lower salt concentrations (0.5 – 2% NaCl) but were inhibited in 6% NaCl. Some *S. agalactiae* isolates appeared to have a greater tolerance in the higher salt concentrations (4 – 5%) compared with *S. iniae*. All *S. agalactiae* and *S. iniae* isolates grew at 22, 28 and 37 °C but could not grow at 47 °C. Overall, *S. agalactiae* had the ability to grow at higher temperatures than *S. iniae* whereas *S. iniae* had a greater ability to grow at lower temperatures.

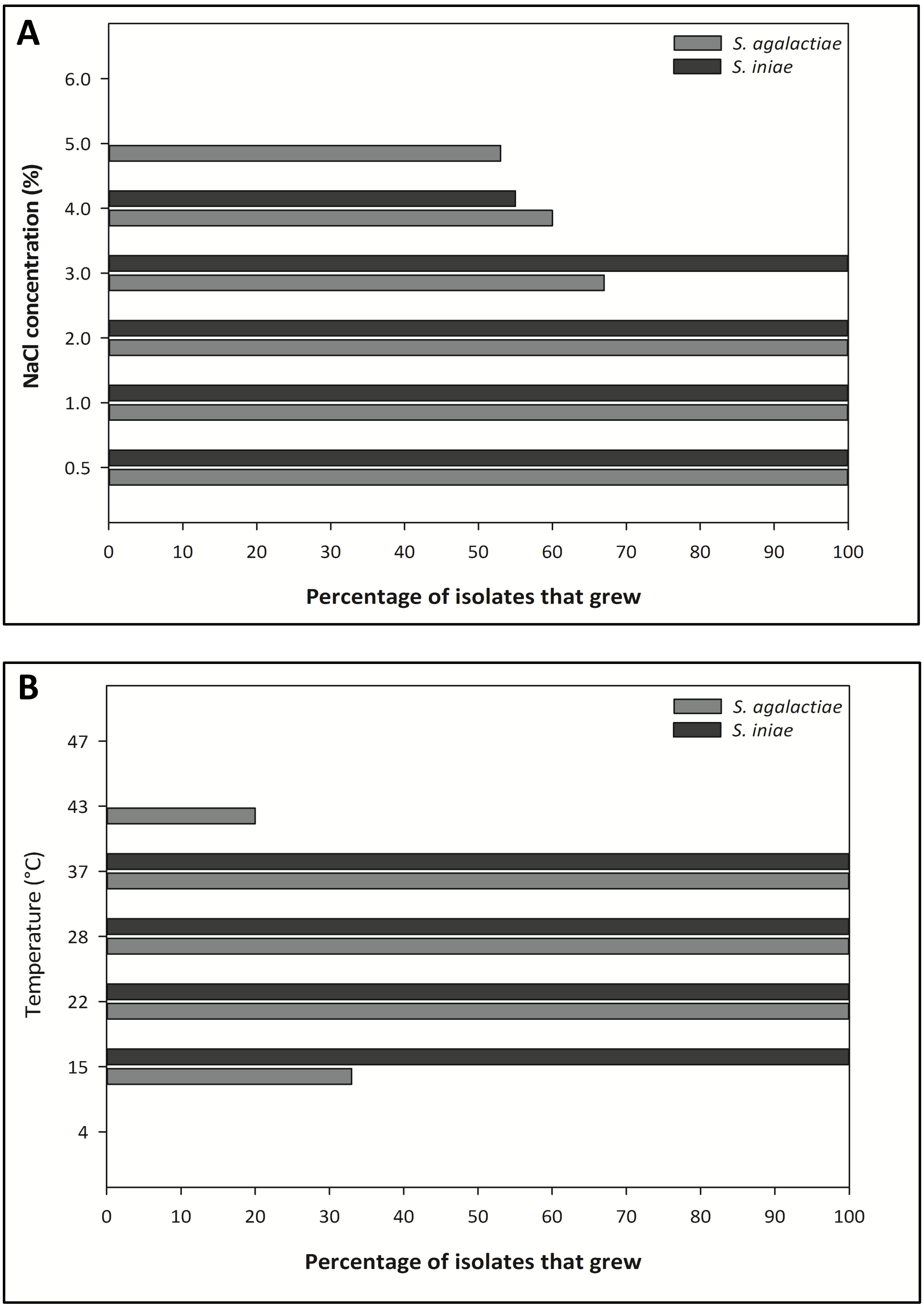

Figure 2.1 The total percentage of *Streptococcus agalactiae* (n = 15) and *Streptococcus iniae* (n = 11) isolates with viable growth in different [A] salt concentrations and [B] temperature ranges

2.3.2 Bacterial identification from biochemical tests

The API 20 STREP profiles gave a range of results with higher variation observed between the *S. agalactiae* isolates than *S. iniae*. Several biochemical profiles were found to be consistently positive or negative across all the isolates examined in this study although variation was observed for numerous biochemical readings for each bacterial species (Table 2.3).

The API 20 STREP results from this experiment were compared with those published by other authors as shown in Table 2.3. Inconsistencies between different publications regarding API 20 STREP results were common. In all the literature reports *S. agalactiae* strains tested positive for alkaline phosphatase and negative for aesculin, pyrrolidonylarylamidase, β galactosidase, arabinose, mannitol, sorbitol, inulin, raffinose, amygdalin and glycogen. All *S. iniae* strains tested positive for pyrrolidonylarylamidase, alkaline phosphatase and leucine arylamidase but negative for Voges-Proskauer, hippurate, sorbitol, arabinose and lactose.

Table 2.3 Summary of API 20 STREP results for *Streptococcus agalactiae* and *Streptococcus iniae* aquatic isolates found in this study and in other literature reports

	S. agalactiae										***S. iniae***									
Reference*	A	B	C	D	E	F	G	H	I	J	A	G	K	L	M	N	O	P	Q	R
Number of isolates	15	8	4	8	20	20	10	20	1	9	11	10	10	1	3	65	26	11	24	31
VP	100	n/a	0	0	100	100	100	100	100	100	0	0	0	0	0	0	0	0	0	0
HIP	67	100	100	100	100	0	0	0	100	100	0	0	0	0	0	0	0	0	0	0
ESC	0	0	0	0	0	0	0	0	0	0	100	100	100	100	100	3	100	100	100	100
PYRA	0	0	0	0	0	0	0	0	0	0	100	100	100	100	100	100	100	100	100	100
α-GAL	13	100	0	0	0	0	0	100	0	0	45	0	60	0	0	0	0	0	0	0
β-GUR	7	100	0	0	70	0	0	0	0	0	91	100	100	100	0	2	100	100	100	100
β-GAL	0	0	0	0	0	0	0	0	0	0	27	0	40	0	100	0	0	0	0	0
PAL	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
LAP	67	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
ADH	53	100	100	100	100	100	100	100	100	33	0	100	100	100	100	11	100	27	v	100
RIB	60	100	100	100	100	100	100	100	n/a	89	100	100	100	100	100	77	100	100	100	100
ARA	0	0	0	0	0	0	0	0	n/a	0	0	0	0	0	0	0	0	0	0	0
MAN	0	0	0	0	0	0	0	0	0	0	18	100	100	100	100	69	100	100	100	100
SOR	0	0	0	0	0	0	0	0	n/a	0	0	0	0	0	0	0	0	0	0	0
LAC	7	100	0	0	0	0	0	0	n/a	0	0	0	0	0	0	0	0	0	0	0
TRE	47	100	100	100	45	0	0	100	0	89	100	100	100	100	100	95	100	100	100	100
INU	0	0	0	0	0	0	0	0	n/a	0	9	0	0	0	0	0	0	0	0	0
RAF	0	0	0	0	0	0	0	0	n/a	0	9	0	0	0	0	0	0	0	0	0
AMD	0	n/a	0	0	0	0	0	0	n/a	0	100	0	100	0	100	74	100	100	100	100
GLYG	0	0	0	0	0	0	0	0	n/a	0	82	100	100	100	100	57	100	100	0	100

[A] Current study [B] Wang *et al*., 2013 [C] Geng *et al*., 2012 [D] Duremdez *et al*., 2004 [E] Al-Marzouk *et al*., 2005 [F] Salvador *et al*., 2005 [G] Eldar *et al*., 1994 [H] Maisak *et al*., 2008 [I] Azad *et al*., 2012 [J] Oanh and Phuong, 2012 [K] Suanyuk *et al*., 2010 [L] Aamri *et al*., 2010 [M] Yuasa *et al*., 1999 [N] Nho *et al*., 2009 [O] Zhou *et al*., 2008 [P] Colorni *et al*., 2002 [Q] Nawawai *et al*., 2008 [R] Bromage *et al*., 1999.

(VP) Voges-Proskauer; (HIP) Hippurate; (ESC) Aesculin; (PYRA) Pyrrolidonylarylamidase; (αGAL) α galactosidase; (βGUR) β glucuronidase; (βGAL) β galactosidase; (PAL) Alkaline phosphatase; (LAP) Leucine arylamidase; (ADH) Arginine dihydrolase; (RIB) Ribose; (ARA) Arabinose; (MAN) Mannitol; (SOR) Sorbitol; (LAC) Lactose; (TRE) Trehalose; (INU) Inulin; (RAF) Raffinose; (AMD) Amygdalin; (GLYG) Glycogen.

(n/a) Data not available (v) variable but no value provided. Numbers show percentage of positive strains.

* Positive signs were assumed to indicate that 100% of strains tested were positive and negative signs denoted that 100% of strains were negative.

All the *S. agalactiae* isolates examined in this study were negative for starch hydrolysis whereas all the *S. iniae* isolates were positive for starch hydrolysis. Carbohydrate reactions using sorbitol and amygdalin hydrate were difficult to interpret due to a lack of information regarding the use of appropriate controls. The results of the ADH test were negative for all *S. iniae* isolates, which is in accordance with API 20 STREP findings. The *S. agalactiae* isolates that gave a positive API 20 STREP result for ADH also produced a positive result using conventional methods. An additional 4 *S. agalactiae* isolates were also ADH positive when the conventional method was used (Table 2.4). The VP assay in the API 20 STREP kit was positive for all *S. agalactiae* isolates and negative for all *S. iniae* strains. However, when this test was repeated using conventional methods all isolates gave a negative result compared with the positive control. These results reflect the variablity often reported between the API 20 STREP kit and conventional assays (Table 2.4).

Bacterial growth, with no black diffusible pigment, was observed for all of the bacterial strains tested when cultured on the bile aesculin agar, indicating that these strains were all negative for aesculin hydrolysis. When bile-aesculin discs were used, all *S. iniae* strains were positive and gave a black-brown pigment around the immediate areas of the disc. This was consistent with the positive control sample. *Streptococcus agalactiae* isolates were all negative with no pigmentation around the bile-aesculin discs.

Table 2.4 Comparison between API 20 STREP and conventional method results

	Streptococcus agalactiae		***Streptococcus iniae***	
	API 20 STREP	**Conventional method**	**API 20 STREP**	**Conventional method**
Voges-Proskauer	100	0	0	0
Arginine dihydrolase	53	80	0	0
Sorbitol	0	Inconclusive	0	Inconclusive
Amygdalin hydrate	0	Inconclusive	100	Inconclusive
Aesculin	0	0 (agar) 0 (disc)	100	0 (agar) 100 (disc)

Numbers show percentage of positive strains. *Streptococcus agalactiae* n = 15. *Streptococcus iniae* n = 11.

2.3.3 PCR and gel electrophoresis

Identification of *S. agalactiae* strains was confirmed by the detection of the 270 bp and 220 bp amplification band for all isolates tested using the set of oligonucleotide primers STRA-AgI – STRA-AgII and F1 – IMOD respectively (Figure 2.2). The primer combinations Sin-1 – Sin-2, LOX-1 – LOX-2 and SP-1 – SP-2 all gave a single and specific amplification product for *S. iniae* strains; 870 bp, 300 bp and 377 bp respectively (Figure 2.3). The primer set designed by Berridge *et al.* (1998) was unsuccessful in identifying any *S. iniae* isolate tested including the positive control. All PCR assays included controls, which were positive for the correct molecular weight band for each primer set tested. Furthermore negative controls, consisting of both recognised culture collections and Milli-Q water (no DNA), showed no amplified products. The only exceptions to this was the F1/IMOD primer set gave an indistinct 220 bp band for the negative control samples *E. faecalis* and *L. garvieae* (Figure 2.2.B). Additionally, a faint band was observed for *E. faecalis* (Figure 2.2.A); however, after the STRA-AgI – STRA-AgII PCR for this isolate was repeated no such banding was observed. This indicates contamination of the original PCR assay for this *E. faecalis* sample.

Analysis of the electrophoresis gels indicated that there were no substantial differences in results between individual master mix ingredients and the illustra PuReTaq Ready-To-Go PCR Beads in a PCR reaction. This is demonstrated in Figure 2.4 and Figure 2.5 where target amplicons of PCR amplification were present at expected band levels. In Figure 2.5.B, when a master mix was used, there is improved clarity as there appears to be a reduced ‘smudged’ appearance to the bands, more specifically in Lane 1. In Figure 2.5.A where the Ready-To-Go PCR Beads were utilised, the banding in Lane 3 is more visually distinguishable in comparison. Nevertheless, both PCR methods tested in this study appeared suitable for identification purposes.

The duplex PCR assay resulted in the amplification of a 870 bp band for *S. iniae*, a 220 bp band for *S. agalactiae* and two bands at 870 bp and 220 bp in the *S. iniae*/*S. agalactiae* mixture. Although the PCR reaction containing a DNA mixture from both bacterial species produced amplified bands, they were of lower visual intensity than when the individual bacteria were used (Figure 2.5).

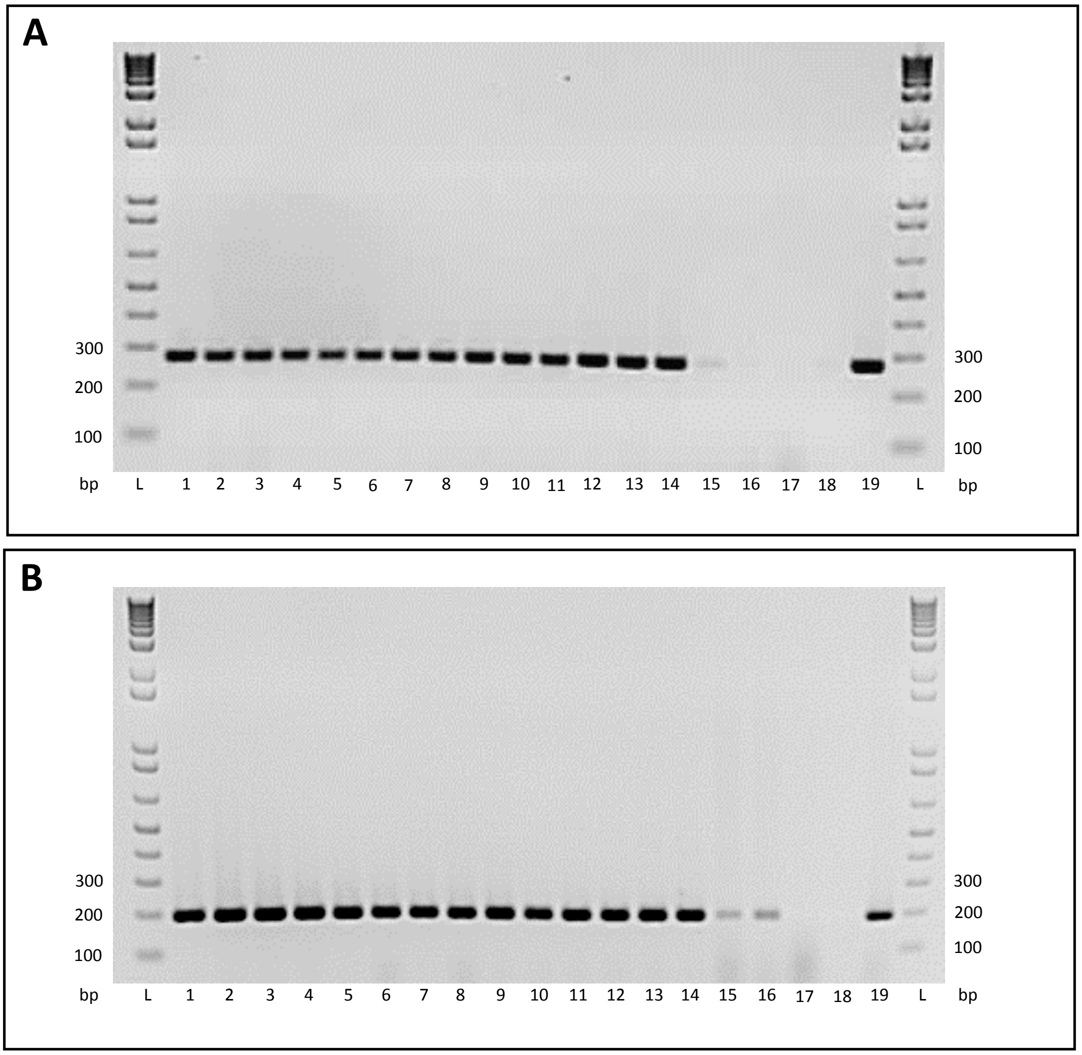

Figure 2.2 Agarose gel showing PCR amplification products using the primer sets: [A] STRA-AgI – STRA-AgII and [B] F1 – IMOD. L denotes 1 Kb DNA ladder. [1] *S. agalactiae* B09032 SaTiCr 08-14b [2] *S. agalactiae* B09032 SaTiBe 08-18a [3] *S. agalactiae* B09032 SaTiBe 08-18b [4] *S. agalactiae* B09032 SaTiBe 08-21a [5] *S. agalactiae* B09032 SaTiBe21b [6] *S. agalactiae* B08059 28E [7] *S. agalactiae* 09011056/1L [8] *S. agalactiae* 09011088/2L [9] *S. agalactiae* May 06 – 6 [10] *S. agalactiae* K0101 [11] *S. agalactiae* K0102 [12] *S. agalactiae* K0103 [13] *S. agalactiae* K0104 [14] *S. agalactiae* K0105 [15] negative control *E. faecalis* NCIMB 775 [16] negative control *L. garvieae* NCIMB 702155 [17] negative control *S. iniae* ATCC 29178 [18] negative control Milli-Q water and [19] positive control *S. agalactiae* NCIMB 701348**.**

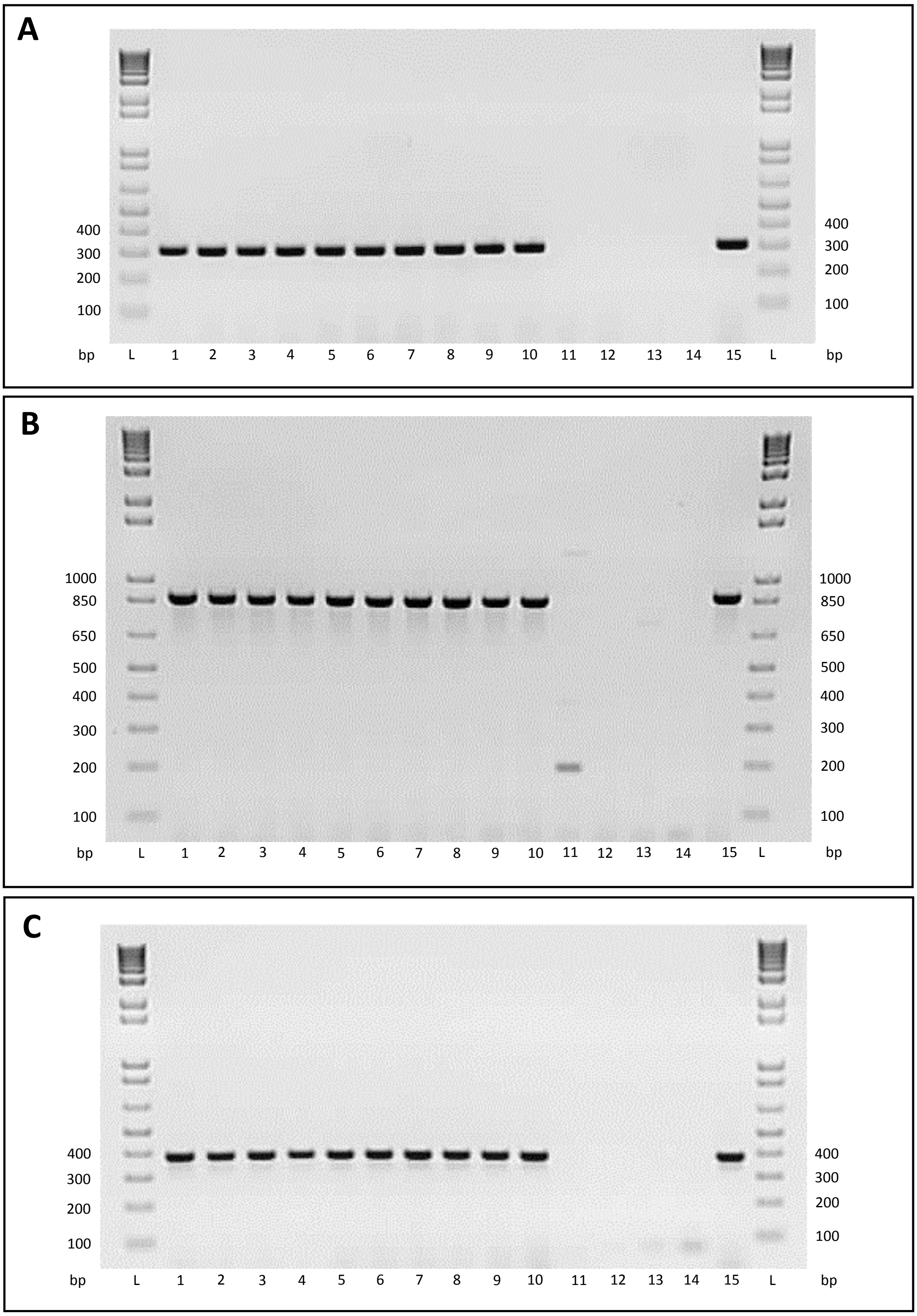

Figure 2.3 Agarose gel showing PCR amplification products using the primer sets: [A] Sin-1 – Sin-2 [B] LOX-1 – LOX-2 and [C] SP-1 – SP-2. L denotes 1 Kb DNA ladder. [1] *S. iniae* B99115 [2] *S. iniae* B99120 1090/99 [3] *S. iniae* B99120 1103/99 [4] *S. iniae* B99120 1104/99 [5] *S. iniae* B99120 1105/99 [6] *S. iniae* B99120 1121/99 [7] *S. iniae* B99120 1121/99 Parrotfish [8] *S. iniae* B99130 B [9] *S. iniae* B99130 K [10] *S. iniae* J39 [11] negative control *E. faecalis* NCIMB 775 [12] negative control *L. garvieae* NCIMB 702155 [13] negative control *S. agalactiae* NCIMB 701348 [14] negative control Milli-Q water and [15] positive control *S. iniae* ATCC 29178.

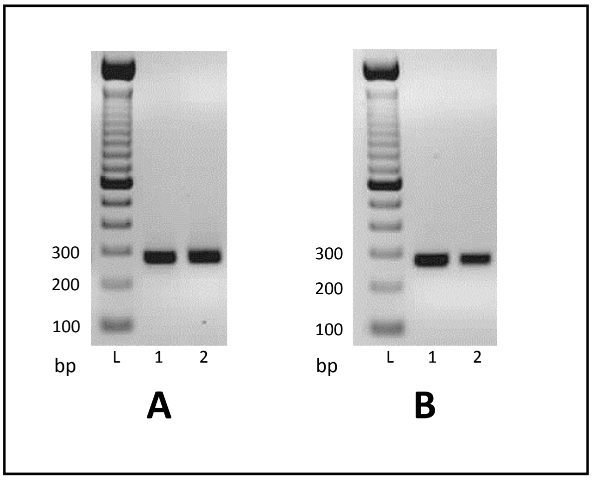

Figure 2.4 PCR amplification of *Streptococcus agalactiae* DNA using STRA-AgI – STRA-AgII primers. L denotes 1 Kb DNA ladder. [A] PCR using illustra PuReTaq Ready-To-Go PCR Beads and [B] PCR using a master mix [1] *S. agalactiae* NCIMB 701348 and [2] *S. agalactiae* 09011088/2L.

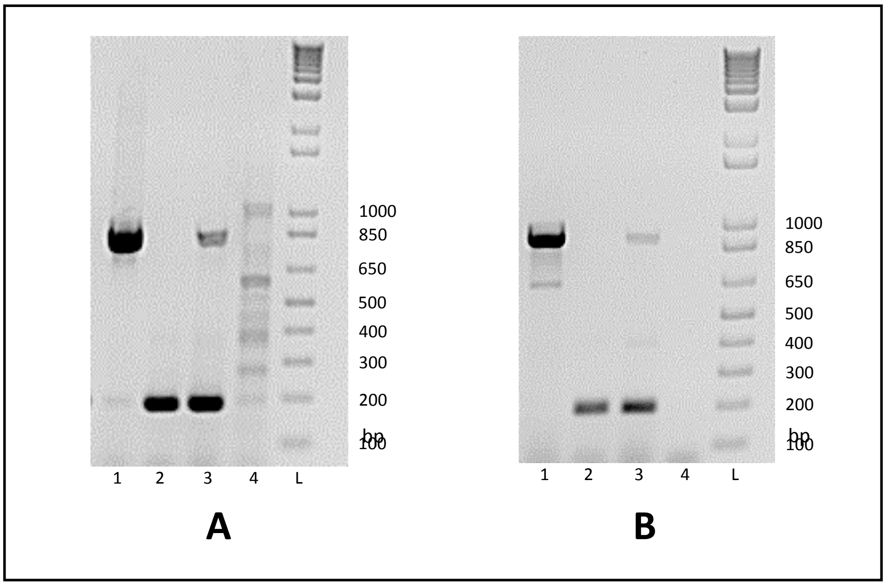

Figure 2.5 Agarose gel showing amplification products from a duplex PCR using the primers LOX-1 – LOX-2 and F1 – IMOD. L denotes 1 Kb DNA ladder. [A] PCR using illustra PuReTaq Ready-To-Go PCR Beads and [B] PCR using a master mix [1] *S. iniae* [2] *S. agalactiae* [3] *S. iniae* and *S. agalactiae* mixture and [4] negative control Milli-Q water.

2.4 Discussion

The bacterial species *S. agalactiae* and *S. iniae* can be found concurrently within the same culture system and it has been noted that a disease outbreak from one of these pathogens can be followed by secondary outbreaks caused by the other bacterial species (Conroy *et al*., 2009). Identification and differentiation of these streptococcal species is fundamental in disease management and prevention, however due to the heterogeneous nature of each subspecies this has proved problematic. This is largely due to the phenotypic diversity observed within strains of the same species, thought to be a result of selection pressure driven by vaccine programmes. For instance, in an attempt to manage *S. iniae* outbreaks, vaccine development and application has inadvertently resulted in the emergence of novel serotypes. It was found that long-term vaccination of rainbow trout against serotype I (ADH positive) *S. iniae* resulted in the emergence and outbreak of vaccine-resistant serotype II (ADH negative) *S. iniae* strains (Bachrach *et al.*, 2001; Eyngor *et al*., 2008). In this case, it was suggested that selective pressure within a closed community may have enabled the pathogen to acquire novel pathogenic mechanisms thus demonstrating the pathogens process of adaptation (Eyngor *et al*., 2008).

It is necessary to recognise variability amongst bacterial isolates and understand the repercussions this may have on bacterial identification. Within this study a selection of identification and characterisation tests were performed to investigate their abilities and limitations in confirming isolates to genus *Streptococcus* and then to differentiate between *S. iniae* and *S. agalactiae*. It was found that *S. iniae* and *S. agalactiae* appear to be visually indistinguishable in their colony morphologies on SBA and TSA. Edwards medium is considered to be selective for the rapid isolation of *S. agalactiae* and other streptococci associated with bovine mastitis. The components crystal violet and thallium salts within the medium serve as the selective agents for presumptive recovery of streptococci. The additional aesculin within the Edwards medium should allow differentiation of aesculin-positive streptococci (Group D *Streptococcus*) through the formation of black colonies, from aesculin-negative streptococci (*S. agalactiae*) through the formation of blue to colourless colonies. In this study, it was hypothesised that since all *S. agalactiae* were aeculin-negative and all *S. iniae* isolates aesculin-postive, on an API 20 STREP that this medium would be able to differentiate between these two species. However, all of these isolates grew as blue colour colonies on this medium so the ability of *S. iniae* to breakdown aesculin may depend on the method used. Therefore, although this growth medium can identify species from the *Streptococcus* genus it cannot distinguish between them, in particular between *S. agalactiae* and *S. iniae*. Thus, showing that whilst this agar may be useful in field recovery, it has limited value for differentiating the two streptococcal species.

Other selective agars for the isolation and detection of Group B streptococci (GBS) from human clinical specimens have been developed (Oxoid Ltd, Basingstoke, UK and BD, Heidelberg, Germany). The orange colony colouration formed when *S. agalactiae* is grown on these selective mediums is claimed to be highly specific. Research has shown that the genetic basis for the haemolysin and pigment production of *S. agalactiae* is linked (Spellerberg *et al*., 1999). Consequently, these specialised growth mediums are unsuitable for *S. agalactiae* fish isolates where a high proportion of the strains recovered from clinical disease outbreaks are non-haemolytic (Sheehan, 2009). It would appear therefore, that whilst some selective agars might help with the initial pathogen recovery, there is no commercially available selective medium that can differentiate between the two aquatic pathogens *S. agalactiae* and *S. iniae*.

The general consensus in the published literature is that *S. agalactiae* strains vary in their ability to haemolyse blood, which was supported by the findings presented here. In this study bacteria were categorised as haemolytic or non-haemolytic and were not differentiated into the common classifications as beta-, alpha- or gamma-haemolytic. The reason for this was that some researchers, such as Facklam (2002), believe there are no benefits to gain from using the beta-, alpha- or gamma-classifcation system. Facklam (2002) stated that 'there was no documented enzyme or toxin that affects red blood cells to produce an alpha-haemolysis by streptococci'. In addtion, haemolysis categorisation is complicated by environmental conditions that may impact growth and appearance of the bacterium making classifications less informative and reliable. Alpha-haemolysis, for example, is categorised by the obsevation of green colouration around the bacterial colonies (Buller, 2004) caused by the production of hydrogen peroxide. However, if streptococcal bacterial are grown in anaerobic conditions the peroxide is not produced therefore under anaerobic conditions alpha-haemolytic isolates may be mistaken as being non-haemolytic or gamma-haemolytic (Facklam, 2002).

Regardless of these complications in haemolysis classification, the haemolytic ability of bacterial strains has been used to differentiate aquatic *S. agalactiae* isolates into two distinct clusters or biotypes; a biotype is a variant strain of a bacterial species with distinctive physiological characteristics. Biotype 1 refers to beta-haemolytic *S. agalactiae* strains and Biotype 2 to non-beta-haemolytic strains (Sheehan, 2009). However, visible haemolysis can be subtle, not always clear-cut and consequently haemolytic categories may be unreliable (Cowan and Steel, 2003). Hence this categorisation of *S. agalactiae* into biotypes has yet to be universally implemented when characterising aquatic *S. agalactiae* strains.

One of the earliest methods used for differentiating streptococci was growth tolerance tests (Facklam, 2002). Consequently the salt and temperature ranges used in this study served not to mimic the farming environment but to test the tolerance range and determine the optimal preference for each bacterial species aiding presumptive identification. Previous studies have reported that clinical *S. iniae* infections occur more often when tilapia are reared at lower water temperatures (15 – 24 °C) whereas *S. agalactiae* infections are often associated with higher water temperatures (24 – 28 °C) (Conroy, 2009; Salvador *et al*., 2005).

Findings from this study illustrated that the bacterial species and strains investigated can grow in a wide range of *in vitro* laboratory based temperatures. Such broad temperature extremes are not unusual for aquatic bacterial species as most bacterial species display growth in wide temperature tolerances. However, it was found that aquatic strains of *S. agalactiae* had a wider temperature and salt tolerance range compared with *S. iniae*. Additionally, there was higher intra-species variation in *S. agalactiae* strains regarding their ability to grow in a range of culture conditions. This cumbersome classification system has now been replaced with more specific and informative procedures and consequently is now not routinely used for identification purposes. Nevertheless, temperature and salinity tolerance assays can provide valuable information on strain variability in survival and growth within different environmental conditions and during a clinical disease outbreak.

Evaluation of the API 20 STREP system indicated that there were phenotypic differences between isolates of the same bacterial species. Given the previous scientific literature this was not unexpected but it does make a reliable single biochemical profile for these two pathogens almost impossible to achieve. The variability of API 20 STREP assays means that identification of such species through this method is complicated and could potentially be a significant cause of misidentification. Additionally *S. iniae* has not been listed in API 20 STREP systems, this may also cause misidentification or unidentified organisms being specified as the pathogen (Facklam *et al.*, 2005; Lau *et al.*, 2006). This was shown when reports of human infections caused by *S. iniae* were misidentified as *S. uberis*, *S. dysgalactiae* and *S. anginosus* by commercial identification systems (Lau *et al*., 2006; Nawawi *et al*., 2008). Further complications arise with the use of commercial kits as there is no standardised procedure for their use in identifying aquatic bacteria. Whilst advice is provided by the manufacturer, often this advice is for mammalian isolates and not applicable to aquatic strains. For example, some research studies have deviated from manufacturer's instructions that suggest an API 20 STREP incubation temperature of 36 °C. Since some aquatic bacterial strains cannot grow at 36 °C (Delannoy *et al*., 2013), researchers have opted for alternative incubation temperatures of 24 °C (Colorni *et al.*, 2002), 27 °C (Bromage *et al*., 1999) or 30 °C (Eldar *et al.*, 1995; Salvador *et al*., 2005; Yuasa *et al.*, 1999). Other factors known to affect results obtained from API 20 STREP include variations in the bacterial cell concentrations of the inoculum used (Barnes and Ellis, 2003), different incubation temperatures (Vandamme *et al.*, 1997), different incubation times and the obvious drawback that interpretation of these colorimetric reactions is semi-quantiative (Evan *et al*., 2004). Nevertheless, the individual assays within the API 20 STREP system can still be utilised to aid identification (Agnew and Barnes, 2007). In this study it was found that particular tests within the API 20 STREP showed consistent results throughout all the isolates investigated or showed distinct differences between *S. agalactiae* and *S. iniae* strains. However, when compared with the literature only the pyrrolidonylarylamidase showed a difference between *S. agalactiae* and *S. iniae* strains.

In this study, the VP reaction tested positive for *S. agalactiae* strains but negative for *S. iniae* on an API 20 STREP and therefore appeared to be a suitable presumptive identification test to distinguish between these two species. However, as found in other studies (Evans *et al.*, 2002) *S. agalactiae* isolates in an API system showed a positive VP reaction whilst conventional tests gave a negative result. It is undetermined which method provides a precise result regarding the bacteria's ability to produce acetoin from glucose fermentation. Differences between results from API 20 STREP and conventional methods have been observed previously, as Barnes and Ellis (2003) found that variation in ADH activity in *S. iniae* may be an artefact of the assay. Additionally, difficulty interpreting results from carbohydrate reactions (sorbitol and amygdalin hydrate) in this study suggested that further use of these tests for identification purposes is unwarranted until appropriate protocols can be established.

All strains of the *S. iniae* and *S. agalactiae* isolates examined in this study were unable to hydrolyse aesculin when using the bile aesculin agar method, which is in agreement with other studies (Conroy, 2009). However once again, different results were obtained when different methods were used, in this case when the bile-aesculin disk and the bile aesculin agar methods were compared. The disc method showed a distinct difference between *S. agalactiae* and *S. iniae* where all *S. iniae* strains tested positive and all *S. agalactiae* strains negative. The reason for this disparity in results between these two methods was not clear. Nevertheless, the results presented clearly demonstrated that *S. agalactiae* and *S. iniae* strains can be distinguished from each other by selected phenotypic properties; in particular their ability to hydrolyse aesculin using the disc diffusion method. Distinction between these two pathogens can also be seen through a starch hydrolysis test. Starch hydrolysis testing has been incorporated into previous *S. iniae* identification procedures (Shoemaker *et al*., 2001) as it is claimed that this is one of the few streptococcal species capable of hydrolysing starch. However, it has been reported that the starch reaction within an API 20 STREP, starch acidification, has been frequently mistaken for starch hydrolysis which is a separate biochemical reaction and one which other streptococcal species test positive for (Evan *et al*., 2004). Although a clear distinction was apparent in the ability of all the *S. iniae* and *S. agalactiae* isolates to hydrolyse starch in this study other research has shown variability of *S. agalactiae* isolates in starch hydrolysis (Evans *et al*., 2004). Evans *et al*. (2004) found that the vast majority of *S. agalactiae* samples from fish, human and bovine sources were negative for starch hydrolysis but strain variation and incubation temperature had the ability to alter the hydrolysis reaction and produce positive results. Consequently, starch hydrolysis would need to be supplemented with additional tests for the distinctions to be made between the *S. iniae* and *S. agalactiae* isolates. Furthermore, the tests for aesculin and starch hydrolysis take the equivalent amount of time to perform as API 20 STREP and are overall less informative.

Although API 20 STREP results show intra-species variation looking at particularly individual results, as mentioned previously, will aid the differentiation between *S. agalactiae* and *S. iniae*.

The classification and identification of streptococci has been routinely aided through serologically identifying polysaccharide group antigens of *Streptococcus* (Cowen and Steel, 2003). Lancefield serotyping describes 20 serotypes of streptococci named Lancefield Groups A-H, K-V. *Streptococcus agalactiae* is the only *Streptococcus* species that has the Group B antigen. This is consistent with findings from this study where results using the Slidex Strepto-kit only provided positive results for *S. agalactiae* isolates. It has been reported however that some other streptococcal species cross-react with commercial slide agglutination tests such as *S. porcinus* (Facklam, 2002). This highlights the importance of performing an array of identification techniques and not to solely rely on one specific assay, as all assays have their benefits and drawbacks.

PCR amplification of DNA sequences with various species-specific primers were used for the definitive confirmation of *S. iniae* isolates. There is a reported lack of specificity in the 16S rRNA primer set designed by Zlotkin *et al.* (1998) between *S. iniae* and *S. difficilis* (*S. agalactiae*) strains (Mata *et al.*, 2004). This has led to the development of additional primer combinations such as LOX-1 – LOX-2, which are considered to detect *S. iniae* with greater specificity (Mata *et al.*, 2004) compared with other primer sets. The *lctO* gene is very unusual among *Streptococcus* species and consequently is limited to a few species of streptococci and related genera (Gibello *et al.*, 1999). Previous studies have detected *S. iniae* isolates by using both oligonucleotide primers for the 16S gene and the *lctO* gene (Al-Harbi, 2011). However from this study, *S. iniae* strains were identifiable by utilising these primers individually. The specificity of the primers was demonstrated through the addition of three other streptococcal species, where no amplified products were apparent. Although there was a limited number of controls used in this study, the primers nevertheless demonstrated specificity and reliability.

The 16S – 23S rRNA primer set designed by Zhou *et al.* (2011) also successfully identified all *S. iniae* strains examined. However, the primer design 5'144 – 3'516 by Berridge *et al.* (1998) was unsuccessful in identifying any *S. iniae* isolates tested even when this was performed using the positive control reference strain. This finding was also reported by Zhou *et al.* (2011) where *S. iniae* isolates from South China and the reference strain ATCC 29178 could not be detected using this primer set. Primers 5'144 – 3'516 have proved successful in other research studies for example Roach *et al*. (2006) found these primers to give the expected amplicon bands along with the type strain. The success in this study may be due to their thermocycling parameters varying from the conditions recommended from the original Berridge *et al.* (1998) work.

The 16S rRNA and 16S – 23S rRNA primer sets, F1 – IMOD and STRA-AgI – STRA-AgII respectively, successfully identified all *S. agalactiae* isolates. However, for the F1 – IMOD primers *E. faecalis* and *L. garvieae* also showed bands at the same size of *S. agalactiae* isolates. This indicated the lack of specificity in this 16S rRNA primer set, however, if used in conjunction with biochemical assays such as Slidex Strepto-kit it can still be used for differentiation purposes. Both F1 – IMOD and STRA-AgI – STRA-AgII primers were initially designed as a diagnostic tool for mastitis in dairy cattle (Martinez *et al*., 2001; Phuektes *et al.*, 2001), however, through this study it is apparent that the application of these assays may be extended to the identification of *S. agalactiae* from fish samples. References and control strains that were used to demonstrate the specificity of this primer were type strains and small in number. Therefore, if these PCR primer sets were to be used for commercial purposes during clinical outbreaks a more extensive study would be required to demonstrate full validation.

In comparison with the standard master mix PCR protocol, commercially available Ready-To-Go PCR beads were tested and yielded equivalent results. There was an occasional reduced 'smudge' band appearance for master mixes, enabling a more precise estimation of the band size. However, on the other hand amplified products were not as visually distinctive as with the Ready-To-Go PCR beads. Ready-To-Go PCR beads have the additional advantages of minimising risk of contamination due to reduced sample handling and pipetting steps and is robust and reliable. However, it is important to note that DNA is poorly amplified if the Ready-To-Go PCR beads are not completely solubilised before initiating the thermal cycler. Nevertheless, the benefits found with Ready-To-Go PCR beads have justified their continued use in future studies.

Results from the duplex PCR involving LOX-1 – LOX-2 and F1 – IMOD primers indicated that amplification of each primer combination, in particular LOX-1 – LOX-2 was slightly reduced when applied in the duplex PCR assay. This is contradictory to findings from Rodkhum *et al.* (2012) who claimed that there was no deleterious effect of the primers and amplification products when applied in a duplex PCR. Nevertheless, detection of both *S. agalactiae* and *S. iniae* was achieved in this study and therefore a duplex PCR would be useful to simultaneously detect the presence of these two pathogens in a single sample. This would prove beneficial during clinical outbreaks as the clinical signs of these diseases are very similar and both pathogens can be found within the same geographical locations. However, the possible reduced specificity of F1 – IMOD primer means that the duplex PCR would also have to be run in accordance with other biochemical or serological tests to confidently identify *S. agalactiae* samples.

From this study it was apparent that all the primer sets tested, except for 5'144/3'516 primers, are able to identify and distinguish S*. iniae* or *S. agalactiae* isolates. However, for the rapid and specific simultaneous detection of *S. iniae* or *S. agalactiae* from cultures and clinical samples the duplex-PCR will be adopted in future studies. The inclusion of such molecular procedures for initial bacterial identification would be unnecessary however, as assays that assess biochemical and phenotypic characteristics can verify bacterial identification quickly and more economically. Nevertheless, a PCR is essential for definitive identification, especially during simultaneous infection studies.

From the original 18 isolates identified as *S. agalactiae* at the start of this study only 15 were reliably confirmed as *S. agalactiae*. These strains had been initially identified through the veterinary diagnostic facilities at the Institute of Aquaculture, University of Stirling using routine identification and biochemical assays. Similarly, 11 of the 12 *S. iniae* isolates provided were confirmed successfully as *S. iniae*. Isolates that were eliminated from final identification was due to incorrect Slidex Strepto-kit test results for the species. It was prudent not to include strains where there was a conflict between the original and subsequent identification profiles as confidence was required for the differentiation studies, which would have been compromised if only "suspected" strains were used. It is likely that the diagnostic isolates supplied might have been originally misdiagnosed or contaminated during the storage phase. This highlights the difficulty of working and identifying such isolates even within a clinical laboratory.

This study utilised a wide variety of bacterial strains obtained from disease outbreaks from several different geographical locations. This has consequently highlighted the variance in phenotypic characteristics of strains within the same bacterial species and further demonstrated the difficulties of using varied biochemical techniques in characterising and identifying these two bacterial species. It has also been claimed that a key constraint in the identification of *S. agalactiae* and *S. iniae* strains is the lack of clear-cut phenotypic tables in distinguishing bacterial groups from one another (Abbott *et al.*, 2003). Using miniaturised identification systems such as the API 20 STREP system has been noted to have its advantages over conventional testing as preparation costs and the amount of storage space required for reagents can be reduced and identification times can be decreased (Janda and Abbott, 2002). Nevertheless, phenotypic properties of bacteria can be unstable with expression being linked to environmental factors (Janda and Abbott, 2002). Consequently, as highlighted by Abbott *et al.* (2003) repeatability of tests between studies is problematic due to disparities in test conditions. Variables such as growth conditions, medium composition, inoculation procedure and incubation conditions may differ between studies, potentially affecting results (Abbott *et al.*, 2003). Due to the heterogeneous nature of both streptococcal species, the purpose of comparing standard biochemical tests (i.e. API 20 STREP) via conventional laboratory methods was to determine if there was a selection of individual tests that could be performed in a timely and economical manner to identify the two bacterial species and differentiate between them.

Janda and Abbott (2002) stated that the reliance on a single identification system, whether this was phenotypic or genotypic, would provide greater opportunity for misidentifying the bacterial species. Results from the current study supports this and would suggest that once bacteria has been recovered from clinical samples, using SBA or Edwards medium, *S. agalactiae* and *S. iniae* identification must consist of [1] primary assays such as: Gram staining, motility, oxidase test [2] secondary assays such as Slidex Strepto-kit, starch hydrolysis and API 20 STREP test looking particularly at individual results and [3] tertiary assays which involve the duplex-PCR of *S. agalactiae* and *S. iniae* (Table 2.5).

Table 2.5 Summary of assays in recovery and identification of aquatic *Streptococcus agalactiae and Streptococcus iniae*

	Assay	*S. agalactiae*	*S. iniae*
Primary bacterial recovery	SBA or Edwards medium	Haemolysis/no haemolysis Small blue colonies	Haemolysis Small blue colonies
Primary identification techniques	Gram Oxidase Motility	Gram positive cocci Negative Non-motile	Gram positive cocci Negative Non-motile
Secondary phenotypic and biochemical identification	Slidex Strepto-kit Starch hydrolysis API 20 STREP	Positive Negative	Negative Positive
	Positive for:	Alkaline phosphatase	Pyrrolidonylarylamidase, Alkaline phosphatase, leucine arylamidase
	Negative for:	Aesculin, amygdalin, arabinose, β-galactosidase, glycogen, inulin, mannitol, pyrrolidonylarylamidase raffinose and sorbitol	Arabinose, hippurate, lactose, sorbitol and Voges-Proskauer
Tertiary molecular identification	Duplex PCR: LOX-1 – LOX 2 and F1 – IMOD primers	Band at 220 bp	Band at 870 bp

2.5 References

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Chapter 3

Virulence factors of *Streptococcus agalactiae* and *Streptococcus iniae*

3.1 Introduction

It is well established that *Streptococcus agalactiae* and *S. iniae* cause significant morbidity and mortality to a wide variety of fish stocks globally (Evans *et al*., 2006). However, the pathogenicity mechanisms for these two species are not well understood in aquatic strains. The identification and characterisation of virulence mechanisms utilised by these two organisms during an active infection are important in terms of not only understanding the disease and disease progression but also in developing novel diagnostic identification methods and developing new vaccines. Hughes *et al*. (2002) identified several outer surface proteins belonging to human *S. agalactiae* strains that are considered as potential vaccine candidates against *S. agalactiae* infection in humans.

Infections with *S. agalactiae* and *S. iniae* in fish present with a similar clinical pattern, but histopathology descriptions would indicate differences in pathogenesis. To understand the pathogenesis of co-infections any differences between the strains during an infection needs to be determined, and this is likely to be through their expression of virulence factors. To date, the focus of *S. agalactiae* virulence factors has been based on mammalian isolates (human and bovine), which are not necessarily applicable to fish isolates. Previous studies have shown that there can be intra-species variation in relation to the presence of genes coding for particular virulence factors. For example, a study from Duarte *et al*. (2005) looked at the occurrence of four virulence-related genes in bovine and human *S. agalactiae* isolates that regulate the virulence factors: Cβ protein (gene: *bac*), Cα protein (gene: *bca*), Laminin-binding protein (gene: *lmb*) and C5a peptidase (gene: *scpB*). Results indicated that not only was there variation in the occurrence of these virulence genes between isolates obtained from the two different host species but also between bacterial serotypes. Some research has been conducted using fish isolates and again variation in the occurrence of virulence-related genes was apparent between different bacterial strains (Delannoy *et al*., 2013). Furthermore the gene that codes for the virulence factor protein Rib which supports resistance to protease immunity (gene: *rib*), previously found in human *S. agalactiae* isolates (Stålhammar-Carlemalm *et al*., 1993), was absent in all fish isolates tested by Delannoy *et al*. (2013). However, for *S. iniae* several virulence-related genes have been detected in fish isolates (Baums *et al*., 2013). Specifically, there are genes that encode for: phosphoglucomutase (gene: *pgm*), cytolysin SLS (gene: *sagA*), M-Like protein (gene: *simA*), C5α peptidase (gene: *scpI*), capsule (gene: *cpsD*) and polysaccharide deacetylase (gene: *pdi*).

The presence of virulence-related genes does not equate to the expression of determinants by the bacterium. Buchanan *et al*. (2008) employed several *in vitro* assays in an attempt to summarise the key steps in disease progression and determine the role of any virulence factors of *S. iniae*. By utilising bacterial strains of different pathogenic abilities Buchanan *et al*. (2008) was able to identify that neither the adherence to and invasion of cells nor the resistance to antimicrobial peptides by *S. iniae* were key mechanisms in bacterial invasion during infection in fish. Whereas, the ability of *S. iniae* to avoid phagocytic clearance and oxidative killing proved fundamental in the virulence capability of the bacterial strains.

The aim of this study was to detect specific virulence factors associated with virulent strains of *S. agalactiae* and *S. iniae* recovered from fish. *In vitro* assays were used to not only assess the presence or absence of particular virulence-associated genes but also the expression of particular virulence factors. This study was intended to identify the virulence capability of a range of aquatic *S. agalactiae* and *S. iniae* strains by passaging them at high concentrations through Nile tilapia (*O. niloticus*). By making direct comparisons between virulent and avirulent bacterial strains crucial virulent factors could be established. Furthermore, any inter and intra-species variation in the expression of virulence factors could be investigated.

3.2 Materials and Methods

3.2.1 Fish

Nile tilapia (*O. niloticus*) of approximately 5 – 7 months in age and 30 ± 10 g in weight were obtained from in-house stocks at the Tropical Aquarium, Institute of Aquaculture, University of Stirling. All inoculation studies were performed in the Aquatic Research Facility, Institute of Aquaculture, University of Stirling. During the experimental trials fish were kept individually in plastic tanks with continuous flow-through water at a maximum flow of 0.7 L/minute and an air stone used for aeration. Fish were starved for 24 hours prior to inoculation then subsequently fed with a commercial diet from Skretting (Nutra Plus) to apparent satiation twice daily. The light regime used was a 12 hour light: 12 hour dark cycle and the water temperature was maintained at 26 ± 2 °C.

3.2.2 Bacterial passage

As the isolates had been in storage, the aim of this serial passage experiment was to increase the virulence of bacterial pathogens by passaging them at a high concentration through fish. Five isolates of *S. agalactiae* and four of *S. iniae* (Table 3.1) were revived from cryo-beads as previously described (Section 2.2.1). One colony from a pure culture was inoculated into 5 ml tryptone soya broth (TSB) (Oxoid Ltd, Basingstoke, UK) and incubated at 28 °C, 140 rpm (Kühner Shaker ISF-1-W, Adolf Kühner AG, Switzerland) for 24 hours. The bacterial suspension was then centrifuged at 2602 g (MSE Mistral 2000R, MSE, London, UK) for 15 minutes and the supernatant discarded. Five hundred microliters of sterile saline (0.85% NaCl) was added to each bacterial pellet to form a concentrated bacterial suspension. Nile tilapia were injected intraperitoneally (i.p.) with 100 µl of the bacteria suspension and monitored for 48 hours. If any fish showed signs of morbidity during this time they were immediately euthanised with an overdose of 10% benzocaine solution (Sigma-Aldrich, Buchs, Switzerland) and the brain immediately destroyed through dissection.

A swab of the kidney and/or spleen was taken aseptically from any moribund or dead fish and streaked directly onto tryptone soya agar (TSA) (Oxoid Ltd, Basingstoke, UK) plates using a sterile bacterial loop. These inoculated plates were then incubated at 28 °C for 48 hours and checked for bacterial recovery. Any isolates recovered on the TSA were then purified as required and identified using bacterial identification tests including Gram staining, motility, oxidase (Sigma-Aldrich, Buchs, Switzerland) and Slidex Strepto-kit (biomérieux, Marcy l'Etoile, France). Definitive identification was conducted through PCR and gel electrophoresis using LOX-1 – LOX-2 and F1 – IMOD primer sets as earlier described (Section 2.2.5 and 2.2.6). Any surviving fish were sacrificed after 48 hours and treated as described above for bacterial isolation. The *in vivo* passage was repeated a maximum of three times and if after the third passage the bacteria did not cause morbidity or mortality but was successfully recovered, for the purposes of this study, it was considered as avirulent. Once identified, passaged isolates were stored on cryo-beads (Technical Services Consultants Ltd, Heywood, UK) and stored at -70 °C.

Table 3.1 *Streptococcus agalactiae* and *Streptococcus iniae* isolates used for *in vitro* and *in vivo* assays

Bacterial species	Isolate	Source or geographical location	Referred to in text as
Streptococcus agalactiae	09011056/1L	Thailand	*S. agalactiae* A
	09011088/2L	Thailand	*S. agalactiae* B
	May 06 – 6	Vietnam	*S. agalactiae* C
	K0105	Kuwait	*S. agalactiae* D
	B09032 SaTi Cr 08-14 b	Colombia University	*S. agalactiae* E
Streptococcus iniae	B99115	Barbados	*S. iniae* A
	B99120 1103/99	Barbados	*S. iniae* B
	B99130 Kidney	Grenadines	*S. iniae* C
	J39	Korea	*S. iniae* D

3.2.3 Bacterial biochemical profiles

API 20 STREP tests (biomérieux, Marcy l'Etoile, France) were conducted throughout the passage process for each isolate: before the isolate was passaged, after recovery from infected fish and following a short period (< 7 days) in -70 °C storage once recovery of the isolate was made. API 20 STREP assays were conducted according to manufacturer's instructions except for the incubation temperature, which was 28 °C instead of the recommended 36 °C. This test was performed to assess any biochemical changes that may have occurred during bacterial passage.

3.2.4 Bacterial viability counts

Viable cell counts were performed based upon the technique developed by Miles and Misra (1938) to determine the number of viable colony forming units (cfu) in a bacterial suspension. The drop count technique is used in microbiology research laboratories worldwide but has not been standardised. A preliminary study investigating the validation of this method with *Streptococcus* isolates was performed. One colony of *S. agalactiae* B and *S. iniae* C (Table 3.1) recovered from the first passage was placed into 20 ml TSB and incubated in at 28 °C, 140 rpm for 18 hours. After incubation, samples were centrifuged at 2602 g for 15 minutes and the supernatant was discarded. Bacterial pellets were resuspended in saline (0.85% NaCl) and the optical density (OD) of each sample at absorbance 600 nm was measured using a WPA CO 8000 Cell Density Meter (Biochrom Ltd., Cambridge, UK). The bacterial suspension was adjusted using sterile saline to give OD_{600} 1 and OD_{600} 0.5. Ten-fold serial dilutions (10^{-1} – 10^{-7}) of each bacterial suspension (OD_{600} 1 and OD_{600} 0.5) were made and 6 x 20 µl drops were dispensed onto TSA plates. The plates were left to dry at room temperature for 10 minutes and then incubated at 28 °C for 48 hours. The number of cfu per drop was determined using a Stuart cell counter (Bibby Scientific Ltd, Stone, UK) and the number of cfu per ml was calculated. This procedure was repeated in triplicate for both isolates. A cfu count was then performed following this method for the remaining isolates recovered from passage (except *S. agalactiae* E) but without replicates.

3.2.5 Growth curve

Passaged *S. agalactiae* B and *S. iniae* C isolates were revived from a cryo-bead, inoculated onto TSA and incubated at 28 °C for 48 hours. Five colonies from pure growth were then aseptically placed into 12 x 500 ml TSB and incubated at 28 °C at 140 rpm. Samples were taken at 12 time intervals from 0 hours to 96 hours and cfu counts were determined using dilution factors of 10^{-4} – 10^{-7} for each time point. A negative control was used consisting of TSB with no bacterial inoculation.

3.2.6 Haemolysis on sheep's blood agar

The beta-haemolytic activity of the passaged bacterial strains was assessed on sheep blood agar as previously described (Section 2.2.2).

3.2.7 Growth conditions of bacteria for virulence assays (3.2.8, 3.2.9 and 3.2.10)

In all assays, one colony from a pure culture of each bacterial isolate was transferred to 20 ml TSB and incubated at 28 °C, 140 rpm for 18 hours. After incubation, samples were centrifuged at 2602 g for 15 minutes and the supernatant subsequently discarded. To compensate for differences in growth kinetics between the samples, bacterial cultures were then standardized using 0.85% saline by spectrophotometry, OD_{600} 1, unless stated otherwise. In order to estimate the number of bacteria in any sample used, a viable cell count was carried out following the method previously described.

3.2.8 Blood survival assay

Blood survival assays were based on previously published studies (Buchanan *et al.*, 2008; Milani *et al.*, 2010). Fresh heparinised (Sodium heparin, Sigma-Aldrich, St. Louis, MO, USA) fish blood was collected from 4 Nile tilapia (300 – 400 g) via the caudal vessel. Bacterial suspensions of approximately 100 cfu in 100 µl saline (0.85% NaCl), verified by drop counts, were added to 300 µl blood in 2 ml microcentrifuge tubes and incubated at 28 °C, 225 rpm for 1 hour. After incubation, 100 µl aliquots were taken from each sample in duplicate and aseptically plated onto TSA for enumeration of surviving bacteria. A control sample was prepared by inoculating the bacterial strains with saline instead of blood. Survival was calculated as the percentage of bacterial cfu remaining at the end of the assay relative to the number of cfu in the initial inoculum. The bacterial survival assay was performed in triplicate for each bacterial strain tested and repeated five times. Data was analysed using ANOVA followed by Tukey *post-hoc* tests using the statistical software Minitab 16.1.0.

At the end of one assay, one of the three samples (bacteria – blood mixture) for each bacterium was randomly selected for further analysis. Ten microliters of each sample was smeared onto a microscope slide and left to air dry. Slides were then placed in 70% methanol for 3 minutes and stained with either Giemsa staining or Rapid Romanowsky stains. For the Rapid Romanowsky stain, samples were immersed in Rapid Romanowsky solution B and solution C (Raymond A Lamb, Eastbourne, UK) respectively for 30 seconds then rinsed in deionised water (See appendix). For Giemsa staining, slides were immersed in diluted stain (0.5 ml Giemsa in 10 ml deionised water) (VWR International Ltd, Poole, UK) for 10 minutes and then rinsed in deionised water (See appendix). Coverslips were mounted onto slides with Pertex then visualised using a Zeiss AxioCam MRc digital camera on an Olympus BX51 microscope under 100 x magnification.

3.2.9 Haemolysin assay

A haemolysin assay was based on the protocol in Rose and Okrend (1998) and Fuller *et al*. (2002). For the haemoglobin standard curve a range of percentage haemoglobin solutions were created using saline-washed 1% sheep haemoglobin and 1% sheep erythrocyte suspensions (Fisher Scientific UK Ltd, Leicestershire, UK). These solutions were then centrifuged at 600 g for 5 minutes (Sigma 1-14 Microfuge, Sigma, Osterode am Harz, Germany) after which 0.2 ml of the supernatant was transferred into a sterile 96-well microtiter plate. The absorbance of the plate was then read at a range of wavelengths spanning those recommended in the literature: 450 nm, 490 nm, 540 nm, 590 nm and 600 nm (Buchanan *et al*., 2008; Fuller *et al*., 2002; Inglis *et al*., 2008). The absorbance was measured using a Synergy HT multi-mode microplate reader and Gen5 data analysis software (both from BioTek, Potton, UK).

For the haemolysin test, 0.5 ml bacterial suspensions were mixed with a 0.5 ml of a 1% saline-washed sheep erythrocyte suspension. The samples were then incubated at 28 °C for 1 hour followed by 4 °C for 30 minutes. The samples were centrifuged at 600 g for 5 minutes and transferred to a 96-well plate as described previously. The absorbance of the plate was read at 450 nm. A positive haemolysin test is defined as the production of an OD reading equal or above the OD of the 20% haemoglobin standard from the standard curve (Rose and Okrend, 1998). The assay was repeated six times. Erythrocytes suspended in saline plus lysis buffer (See appendix) (complete lysis) or saline alone (no lysis) were used as controls. Data was analysed using ANOVA followed by Tukey *post-hoc* tests.

3.2.10 Complement-mediated killing assay

A complement-mediate killing assay was applied based on the protocol in Buchanan *et al*. (2008). Blood was collected from 4 Nile tilapia (300 – 400 g) via the caudal vessel and allowed to clot at 4 °C for 1.5 hours then centrifuged at 3500 g at 4 °C for 10 minutes. The serum was collected and centrifuged again at 3500 g at 4 °C for 10 minutes. Half of the serum sample was heat inactivated at 60 °C for 30 minutes. Bacterial suspensions of approximately 100 cfu in 100 µl 0.85% saline were added to 100 µl of either 0.85% saline, active or heat-inactivated serum. Samples were incubated at 28 °C for 2 hours after which time a 100 µl aliquot was taken and aseptically plated on TSA for enumeration of surviving bacteria. Survival was calculated as the percentage of bacterial cfu remaining at the end of the assay relative to the starting levels of cfu in the original inoculum. The assay was performed in duplicate for each strain in active and heat-inactivated serum and the experiment was repeated five times. For data analysis the difference in bacterial survival between active and heat inactivated serum was ascertained as follows:

Effect of heat inactivation of serum on bacterial growth = Average survival for bacteria incubated in heat inactivated serum - Average survival for bacteria incubated in active serum

Data was analysed using ANOVA followed by Tukey *post-hoc* tests using the statistical software Minitab 16.1.0.

3.2.11. Determination of capsule presence

Transmission electron microscopy

The presence of a capsule was determined by electron microscopy based on protocols from Hayat and Giaquinta (1970) and Hayat (1986). Colonies of each bacterial isolate taken from a pure culture were transferred into 1 ml sterile distilled water with the bacterial density adjusted to a MacFarland standard of 2. The bacteria were then transferred into 2.5% gluteraldehyde in 100 mM sodium cacodylate buffer (pH 7.2). The samples were then centrifuged at 2602 g for 15 minutes (MSE Mistral 2000R, MSE, London, UK).and the supernatant replaced with fresh 2.5% gluteraldehyde, this was repeated twice. The samples were post-fixed in buffered 1% osmium, washed three times in distilled water and dehydrated in an acetone series at room temperature before being embedded in low viscosity resin. Ultrathin sections on 200-mesh Formvar-coated copper grids were first stained with uranyl acetate followed by Reynold's lead citrate. The sections were observed under an FEI Tecnai Spirit G2 Bio Twin Transverse electron microscope (TEM).

Anthony's capsule stain

The protocol for Anthony's capsule stain was based on that from Hughs and Smith (2007). One colony of each bacterial isolate from a pure culture was placed into 5 ml milk broth (0.95% skim powder milk) and incubated at 28 °C, 140 rpm for 18 hours. After incubation a 20 µl smear of the bacterial suspension was made on a microscope and left to air dry. The slides were immersed in 1% crystal violet for 2 minutes and rinsed with 20% copper sulphate solution (See appendix). *Pseudomonas aeruginosa* ATCC 27853 was used as a positive control and prepared as above with the exception that the bacteria was grown at 22 °C. Coverslips were mounted onto slides using Pertex then visualised using a Zeiss AxioCam MRc digital camera on an Olympus BX51 microscope under 100 x magnification.

3.2.12 Virulence genes of *Streptococcus iniae*: Polymerase chain reaction and gel electrophoresis

Several individual PCR assays were performed to detect genes encoding known virulence factors for *S. iniae*. The associated gene and oligonucleotide primers for the virulence factors phosphoglucomutase, cytolysin SLS, M-Like protein, C5α peptidase, capsule, polysaccharide deacetylase are detailed in Table 3.2 (Baums *et al*., 2013).

DNA was extracted from bacterial samples as previously described (Section 2.2.5). All *S. iniae* samples used in Chapter 2 DNA extraction were utilised in this experiment, *S. agalactiae* NCIMB 701348, *S. iniae* ATCC 29178 and Milli-Q water were used for controls. Each PCR was performed in a 10 µl reaction mixture containing 5 µl of Plain Combi PPP Master Mix (Top-Bio, Jovkova, Prague, Czech Republic), either 120 nM of simAfornew/simArevnew, 100 nM of scpIfor/scpIrev, 100 nM of pgmfor/pgmrev, 100 nM of cpsDfor/cpsDrev, 90nM of pdifor/pdirev or 1000 nM of sagAfor/sagArev (Eurofins MWG Operon, Germany), approximately 100 ng/ µl of template DNA, and Milli-Q water to volume.

DNA amplification was performed in a Biometra thermal cycle (Biometra, Goettingen, Germany). The denaturation, annealing and elongation temperatures and times used were: 94 °C for 1 minute, 55 °C for 30 seconds, 72 °C for 30 seconds for 30 cycles. All amplified products were stored at -20 °C until use.

The amplified products were resolved by gel electrophoresis. Analysis was performed on 1% (w/v) agarose gels, stained with ethidium bromide, using a TAE buffer system (0.5. x Tris-acetate-EDTA) (See appendix). A 3 µl sample of PCR product was mixed with 7 µl of loading buffer (1X BlueJuice gel loading buffer, Invitrogen, Paisley, UK) and 3 µl Milli-Q water. A final 10 µl loading volume was used for all samples. Band patterns were visualised and photographed under UV with the size of the restriction fragments estimated by comparison to a 100 bp DNA ladder with a loading volume of 3 µl (Quick-load 100 bp DNA ladder, New England BioLabs Ltd, Hitchin, UK). Samples were considered positive when a clear band was observed under UV light at the relevant target region for each primer set (Table 3.2.).

Table 3.2 Oligonucleotide primers for virulence genes of *Streptococcus iniae* (Baums *et al*., 2013).

Virulence factor	Gene	Primer	Nucleotide sequence (5'-3')	Target region (bp)
M-like protein	*simA*	simAfornew	AATTCGCTCAGCAGGTCTTG	994
		simArevnew	AACCATAACCGCGATAGCAC	
C5α peptidase	*scpI*	scpIfor	GCAACGGGTTGTCAAAAATC	822
		scpIrev	GAGCAAAAGGAGTTGCTTGG	
Phosphoglucomutase	*pgm*	pgmfor	TATTAGCTGCTCACGGCATC	713
		pgmrev	TTAGGGTCTGCTTTGGCTTG	
Capsule	*cpsD*	cpsDfor	TGGTGAAGGAAAGTCAACCAC	534
		cpsDrev	TCTCCGTAGGAACCGTAAGC	
Polysaccharide deacetylase	*pdi*	pdifor	TTTCGACGACAGCATGATTG	381
		pdirev	GCTAGCAAGGCCTTCATTTG	
Cytolysin SLS	*sagA*	sagAfor	AGGAGGTAAGCGTTATGTTAC	190
		sagArev	AAGAAGTGAATTACTTTGG	

3.3 Results

3.3.1 Passage

Four of the five *S. agalactiae* isolates and three out of the four *S. iniae* strains were found to be virulent in Nile Tilapia as determined by the passage studies performed (Table 3.3). All isolates were successfully recovered after passage and conclusively identified as either *S. agalactiae* or *S. iniae* through the described identification techniques (Section 3.2.2)

Table 3.3 The passage of *Streptococcus* strains adminstered *in vivo* to Nile tilapia

Isolate identification	Number of passages performed	Mortality	Virulent or Avirulent
S. agalactiae A	3	No mortality	Avirulent
S. agalactiae B	1	Mortality*	Virulent
S. agalactiae C	3	Mortality*	Virulent
S. agalactiae D	2	Mortality*	Virulent
S. agalactiae E	1	Mortality*	Virulent
S. iniae A	1	Mortality*	Virulent
S. iniae B	1	Mortality*	Virulent
S. iniae C	1	Mortality*	Virulent
S. iniae D	2	No mortality	Avirulent

* Mortality occurred 24 hours post-inoculation

The API 20 STREP profiling of bacteria pre- and post-passage indicated some changes in the regulation of enzyme activity and fermentation of carbohydrates (Table 3.4). In particular, differences were seen in the biochemical profile of some bacterial strains pre-passage and post-passage. The highest degree of variability between pre- and post-passage results was observed in the *S. agalactiae* E strain where the activity of eight biochemical reactions were altered during passage (Table 3.4). For both *S. agalactiae* C and D two changes were observed in α-galactosidase and β-glucuronidase as these were found to be positive for pre-passage bacterium but negative in post-passaged. This suggests that there was reduced activity of these enzymes during passage. For isolate *S. iniae* A, one reaction profile differed during passage, with β-galactosidase becoming negative. Three changes were observed for *S. iniae* B with the activity of α-galactosidase and β-galactosidase ceasing and arginine dihydrolase producing a positive result for the post-passaged bacterium. However, all changes observed in the API 20 STREP profile post-passage reverted to the pre-passage reaction after the bacteria were stored for a short period (Table 3.4). No differences in biochemical reactions were seen for *S. agalactiae* A and B and *S. iniae* C and D at any time throughout the passage process.

Table 3.4 Biochemical profile of *Streptococcus agalactiae* and *Streptococcus iniae* strains pre- and post-passage in fish

		VP	HIP	AES	PYRA	α-GAL	β-GUR	β -GAL	PAL	LAP	ADH	RIB	ARA	MAN	SOR	LAC	TRE	INU	RAF	AMD	GLYG	
	1																					
S. agalactiae A	2	+	+	-	-	-	-	-	+	+	+	+	-	-	-	-	+	-	-	-	-	No change
	3																					
	1																					
S. agalactiae B	2	+	+	-	-	+	-	-	+	+	+	+	-	-	-	-	+	-	-	-	-	No change
	3																					
	1					+	+															
S. agalactiae C	2	+	+	-	-	-	-	-	+	+	+	+	-	-	-	-	+	-	-	-	-	
	3					+	+															
	1					+	+															
S. agalactiae D	2	+	+	-	-	-	-	-	+	+	+	+	-	-	-	-	+	-	-	-	-	
	3					+	+															
	1			-							-	-	-	-			-			-	-	
S. agalactiae E	2	+	+	+	-	-	-	-	+	+	+	+	+	+	-	-	+	-	-	+	+	
	3			-							-	-	-	-			-			-	-	
	1							+														
S. iniae A	2	-	-	+	+	-	+	-	+	+	-	+	-	-	-	-	+	-	-	+	+	
	3							+														
	1					+		+			-											
S. iniae B	2	-	-	+	+	-	+	-	+	+	+	+	-	-	-	-	+	-	-	+	+	
	3					+		+			-											
	1																					
S. iniae C	2	-	-	+	+	-	+	-	+	+	-	+	-	-	-	-	+	-	-	+	+	No change
	3																					
	1																					
S. iniae D	2	-	-	+	+	-	-	-	+	+	-	+	-	+	-	-	+	-	-	+	+	No change
	3																					

[1] Pre-passage [2] Post-passage [3] Post-passage and 7 days after storage. [VP] Voges-Proskauer [HIP] Hippurate [AES] Aesculin [PYRA] Pyrrolidonylarylamidase [αGAL] α galactosidase [βGUR] β glucuronidase [βGAL] β galactosidase [PAL] Alkaline phosphatase [LAP] Leucine arylamidase [ADH] Arginine dihydrolase [RIB] Ribose [ARA] Arabinose [MAN] Mannitol [SOR] Sorbitol [LAC] Lactose [TRE] Trehalose [INU] Inulin [RAF] Raffinose [AMD] Amygdalin [GLYG] Glycogen [+] Positive result [-] Negative result [s] slow reaction. Numbers show percentage of positive strains.

3.3.2 Drop counts

Bacterial colonies were counted in sectors on TSA plates when full-size discrete colonies could be observed over the drop area. Individual colonies could be distinguished from bacterial solutions at both 10^{-6} and 10^{-7} dilution factors. There was variance in drop count values between sectors on the same TSA plate and between replicates (Table 3.5).

Table 3.5 Viable cell counts of a *Streptococcus agalactiae* B and *Streptococcus iniae* C suspension which had been grown for 18 hours at 28 °C with an OD_{600} 1 or OD_{600} 0.5. Values are shown for two different dilution factors which were repeated in triplicate.

***S. agalactiae* B**									
Replicates	OD	Dilution factor	cfu 1	2	3	4	5	6	Ave. cfu
R1	0.94	10^{-7}	4	5	9	3	6	8	6
R2	1.01	10^{-7}	7	12	7	9	8	3	8
R3	1.02	10^{-7}	9	8	5	4	4	2	5
R1	0.94	10^{-6}	38	56	44	50	44	53	48
R2	1.01	10^{-6}	56	47	45	58	56	51	52
R3	1.02	10^{-6}	51	48	55	44	43	54	49
R1	0.50	10^{-7}	2	2	2	4	5	7	4
R2	0.49	10^{-7}	3	3	2	2	2	1	2
R3	0.52	10^{-7}	7	6	3	3	2	2	4
R1	0.50	10^{-6}	33	19	21	32	37	29	29
R2	0.49	10^{-6}	27	27	29	33	28	28	29
R3	0.52	10^{-6}	27	25	30	27	30	20	27

***S. iniae* C**									
Repeats	OD	Dilution factor	cfu 1	2	3	4	5	6	Ave. cfu
R1	1.00	10^{-7}	8	3	3	2	9	10	6
R2	1.01	10^{-7}	10	1	1	5	5	2	4
R3	1.04	10^{-7}	5	5	2	4	3	3	4
R1	1.00	10^{-6}	33	67	69	46	52	72	57
R2	1.01	10^{-6}	35	34	45	34	32	32	35
R3	1.04	10^{-6}	27	24	28	30	28	22	27
R1	0.52	10^{-7}	6	3	4	4	2	5	4
R2	0.52	10^{-7}	1	1	1	2	3	4	2
R3	0.52	10^{-7}	1	1	1	2	3	3	2
R1	0.52	10^{-6}	29	31	40	35	39	34	35
R2	0.52	10^{-6}	20	9	9	16	20	14	15
R3	0.52	10^{-6}	14	15	12	9	7	7	11

From these results, an estimation of the bacterial concentration was made and represented in Figure 3.1. There appears to be less variation between *S. agalactiae* B replicates when the dilution factor 10^{-6} was used whereas the dilution factor 10^{-7} seems to be slightly more preferable for *S. iniae* C than 10^{-6}.

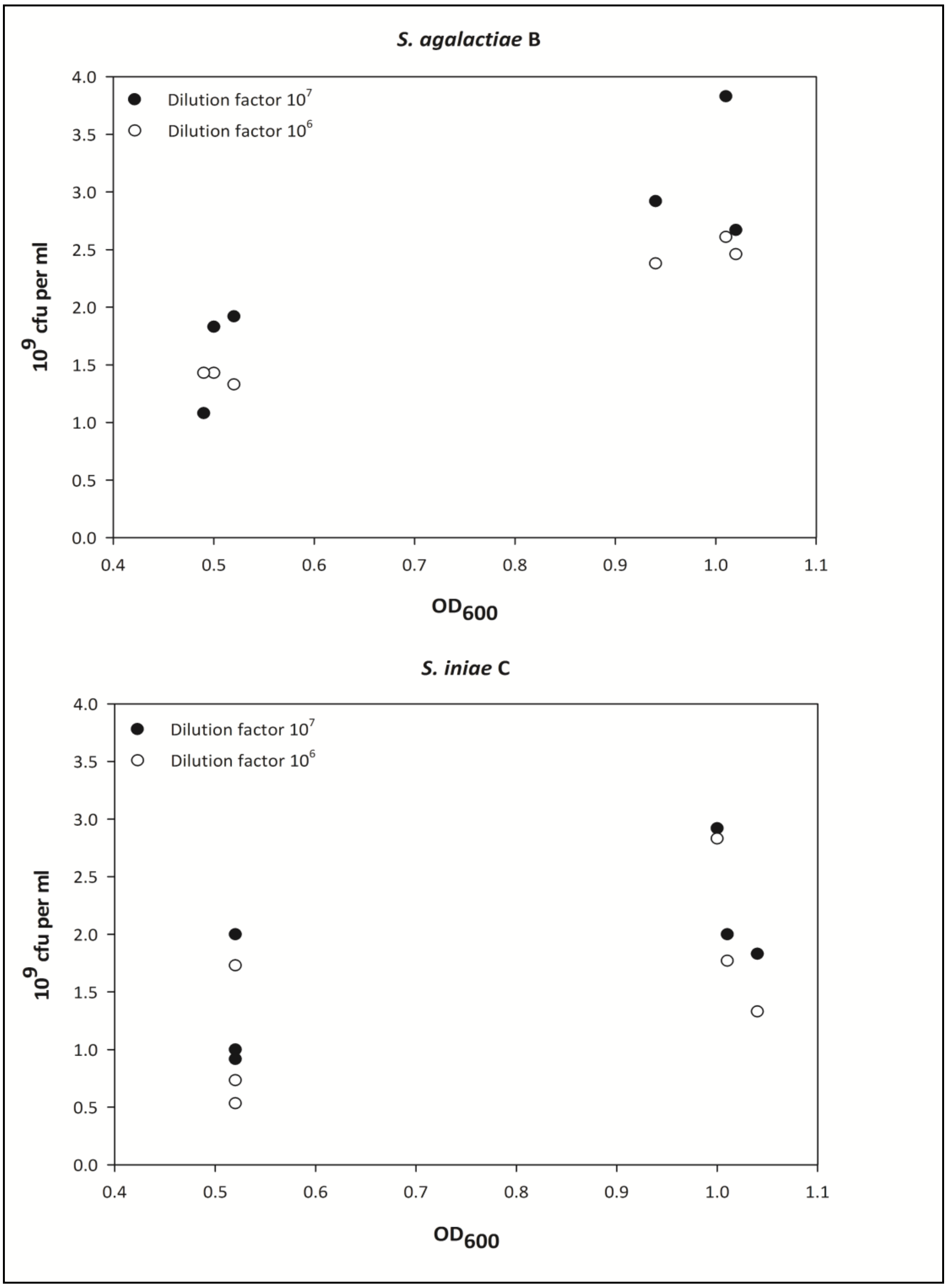

Figure 3.1 Bacterial concentration (cfu per ml) of *Streptococcus agalactiae* B and *Streptococcus iniae* C determined from viable cell counts.

The estimated bacterial concentration of the remaining bacterial strains, which had been previously grown for 18 hours, was calculated using OD_{600} 1 and at the optimal dilution factor as previous described. These results are represented in Table 3.6 and appear to be similar for all the bacterial strains examined.

Table 3.6 Concentration (cfu/ml) of different *Streptococcus agalactiae* and *Streptococcus iniae* isolates calculated from viable cell counts

Isolate	Dilution factor	OD	cfu 1	2	3	4	5	6	cfu per ml
S. agalactiae A	10^{-6}	1.02	16	16	18	20	21	22	9.42×10^{8}
S. agalactiae B	10^{-6}		Ave. taken from Table 3.4.						2.48×10^{9}
S. agalactiae C	10^{-6}	1.02	26	33	40	30	28	38	1.63×10^{9}
S. agalactiae D	10^{-6}	1.00	38	46	38	46	43	37	2.07×10^{9}
S. iniae A	10^{-7}	1.01	1	2	5	7	6	4	2.08×10^{9}
S. iniae B	10^{-7}	1.00	2	2	3	2	3	4	1.33×10^{9}
S. iniae C	10^{-7}		Ave. taken from Table 3.4.						2.25×10^{9}
S. iniae D	10^{-7}	0.99	6	6	3	4	4	4	2.25×10^{9}

3.3.3 Growth curve

The growth of each pathogen was determined by viable cell counts taken from different dilution factors. This provided different values for growth (cfu per ml) at each time point. All data values were plotted to highlight any outliers (Figure 3.2.A and 3.3.A) after which the average bacterial growth was calculated (Figure 3.2.B and 3.3.B). The four different phases of bacterial growth were approximated in Figures 3.2.B and 3.3.B and the results summarised in Table 3.7.

Table 3.7 Time post inoculation of the four different phases of bacterial growth in batch culture for *Streptococcus agalactiae* and *Streptococcus iniae*

	***S. agalactiae* B**	***S. iniae* C**
	Time post inoculation	
Lag phase	0 – 9	0 – 6
Log phase	9 – 18	6 – 18
Stationary phase	18 – 27	18 – 22
Death phase	27 +	22 +

Although these two pathogens reached stationary phase at approximately the same time after incubation, *S. agalactiae* B had a longer lag and stationary phase whereas *S. iniae* C had a longer log phase. Also, as seen in Figure 3.4, *S. agalactiae* B had a considerably higher cfu per ml at each time point than *S. iniae* C; at peak growth *S. agalactiae* B had more than double cfu per ml than *S. iniae* C at its equivalent time point. No bacterial growth was observed in the negative control.

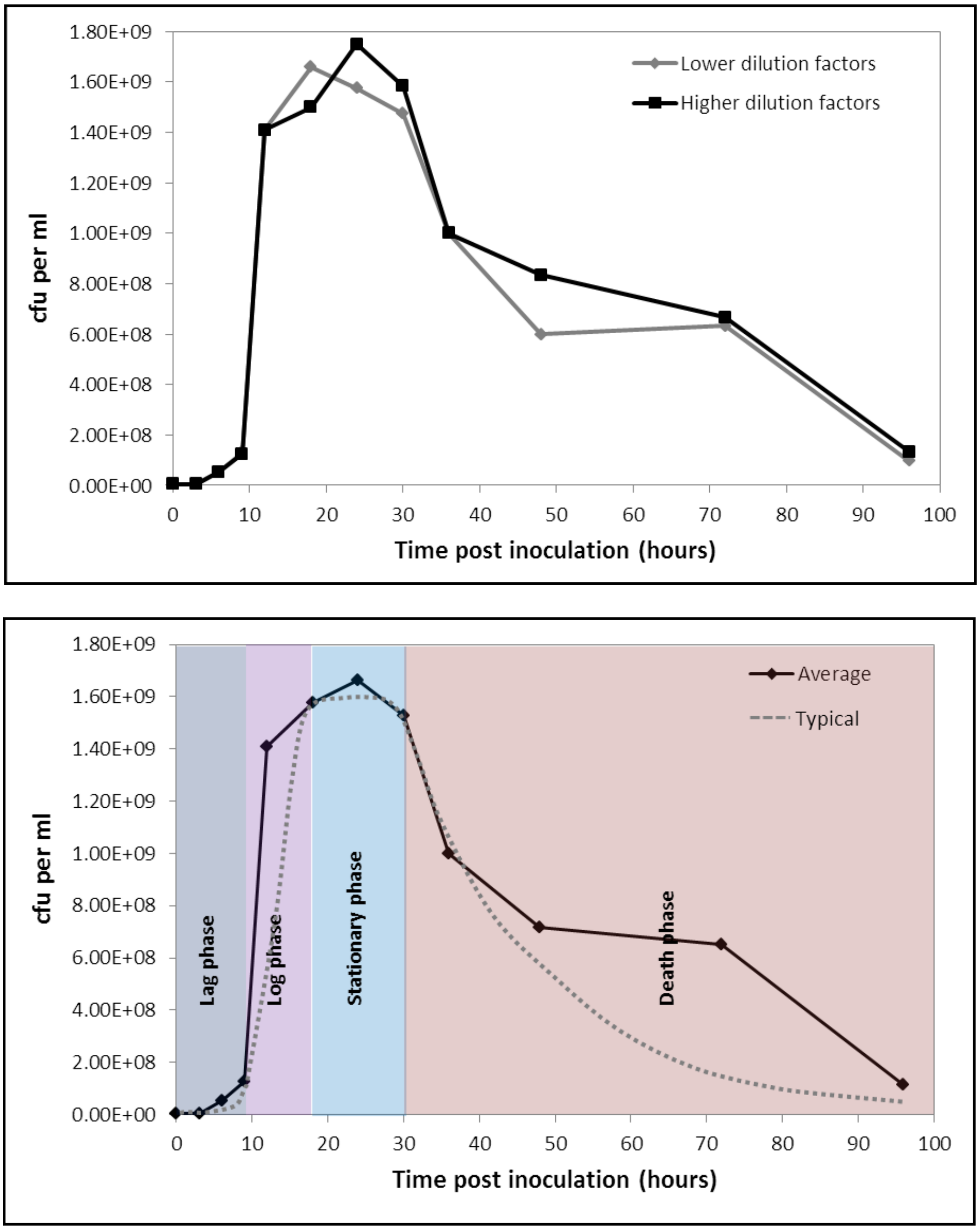

Figure 3.2 *Streptococcus agalactiae* B growth curves. [A] Growth curve of *S. agalactiae* B using values of bacterial concentrations calculated from viable cell counts at different dilutions. [B] Growth curve of *S. agalactiae* B using average values of bacterial concentrations calculated from viable cell counts. A typical bacterial growth curve is included for comparison and to illustrate phases of growth.

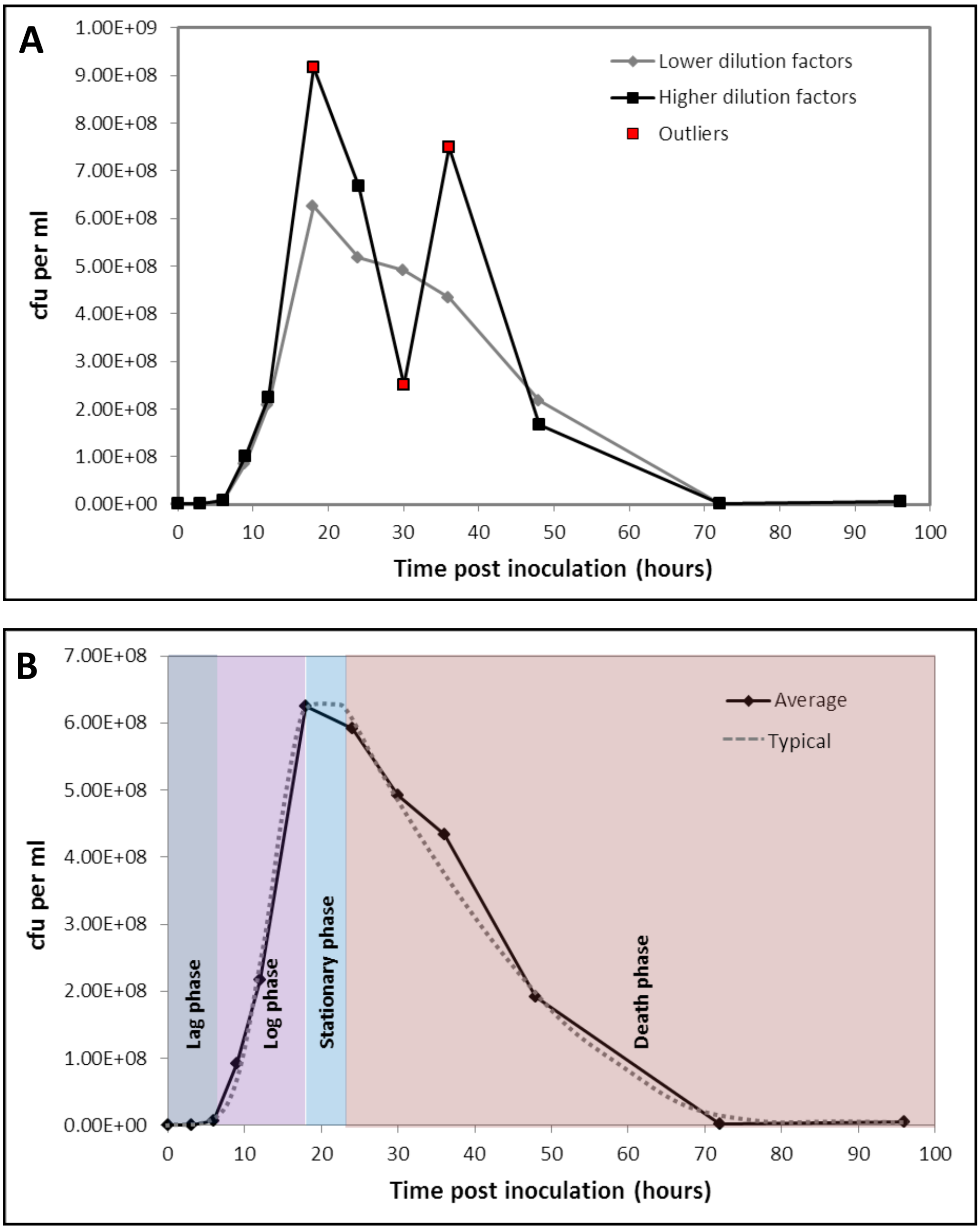

Figure 3.3 *Streptococcus iniae* C growth curves. [A] Growth curve of *S. iniae* C using values of bacterial concentrations calculated from viable cell counts at different dilutions. The data points highlighted in red are outliers. [B] Growth curve of *S. iniae* C using average values of bacterial concentrations calculated from viable cell counts and with the exclusion of outlier values. A typical bacterial growth curve is included for comparison and to illustrate phases of growth.

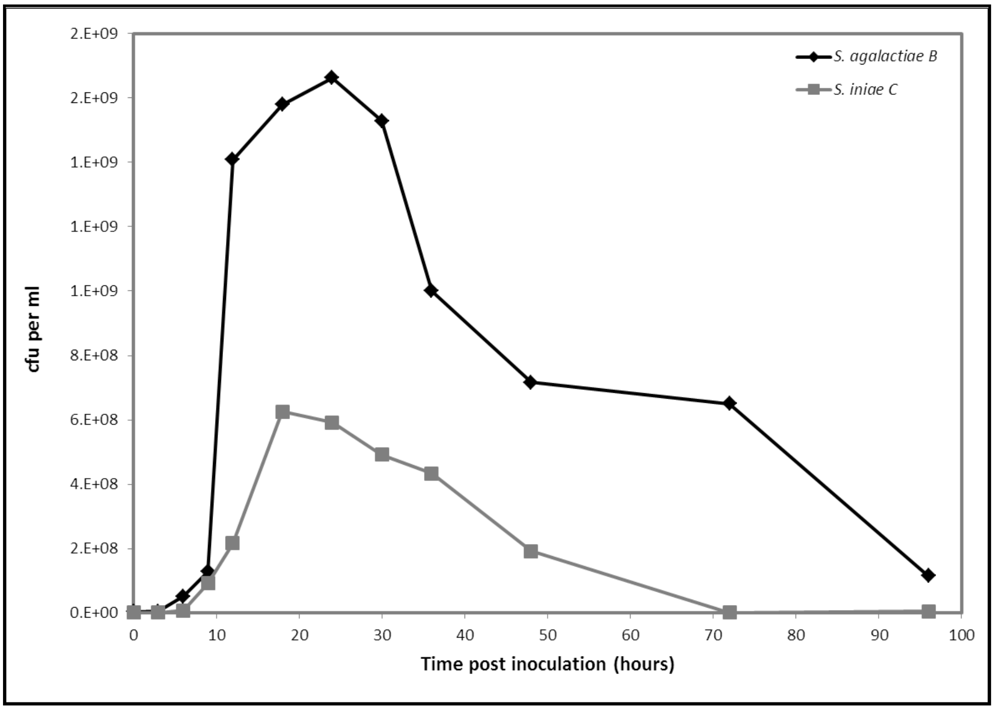

Figure 3.4 Comparison of *Streptococcus agalactiae* B and *Streptococcus iniae* C growth curves using average values from viable cell counts

3.3.4 Haemolysis on sheep's blood agar

No difference was found in the haemolytic ability of the strains tested between pre- and post-passage. All *S. agalactiae* isolates were found to be non-haemolytic and all *S. iniae* isolates were haemolytic (Figure 3.5).

Figure 3.5 Passaged streptococcal isolates after a 48 hour incubation on sheep's blood agar at 28 °C. Top row [left to right]: *S. agalactiae* A, *S. agalactiae* B, *S. agalactiae* C, *S. agalactiae* D. Bottom row [left to right]: *S. iniae* A, *S. iniae* B, *S. iniae* C, *S. iniae* D.

3.3.5 Blood survival assay

This procedure had to be optimised before adequate data could be collected. Initially, the assay was performed using blood from three individual fish (no pooling) and repeated twice. Through a general linear model (GLM) it was found that although there was no significant difference between replicates (p = 0.088). There was, however, a significant difference between individual fish (p = 0.008). To overcome this, blood was pooled from 4 tilapia in subsequent experiments. A paired t-test was performed on the saline controls using before and after incubation data; no significant differences was found between these data groups (p = 0.105) and they were therefore removed from the GLM during further analysis.

The percentage survival of all *S. agalactiae* strains was significantly higher than *S. iniae* when incubated in tilapia blood for 1 hour (p = 0.001). There were significant differences between survival rates from isolates within the same species (Figure 3.6). Nevertheless, loss of virulence was not associated with the ability of *S. agalactiae* or *S. iniae* to survive in blood as there was no significant difference between results obtained from virulent and avirulent isolates. There was a significant difference (p = 0.000) between replicates, however each experiment expressed a distinct pattern of results which was consistent across all the replicates.

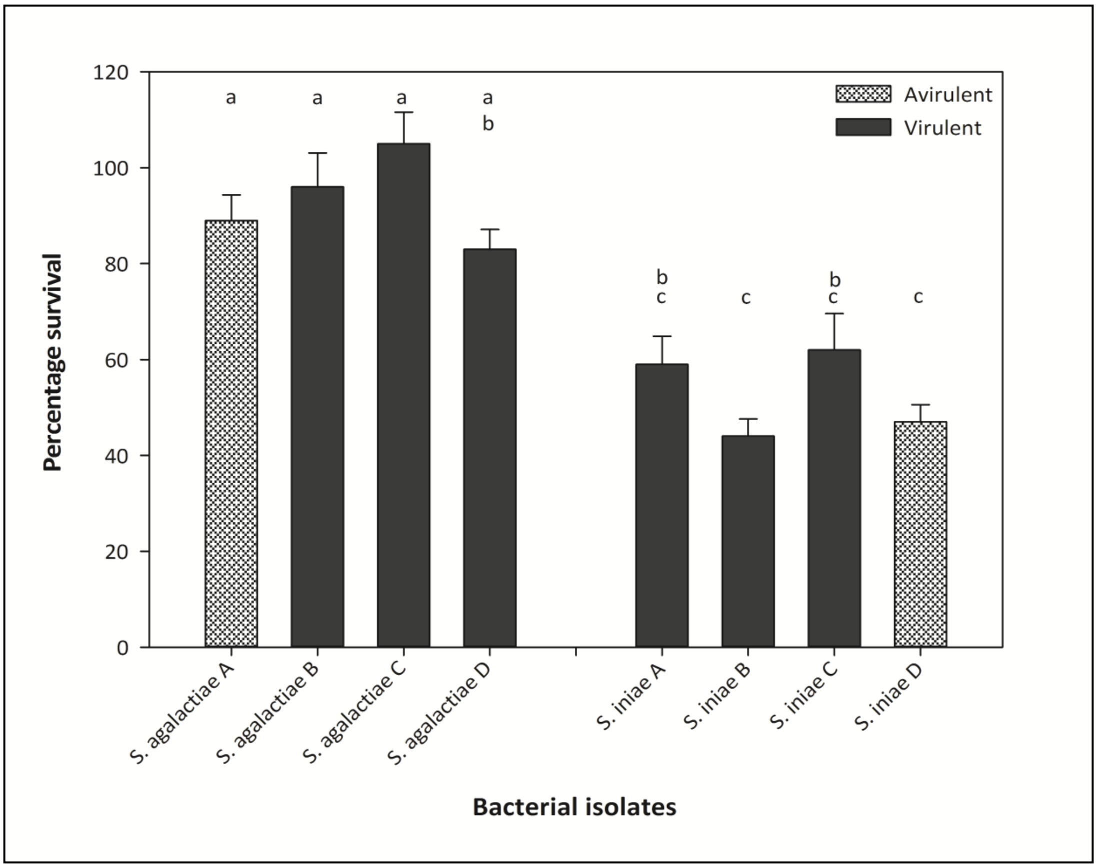

Figure 3.6 Percentage survival of *Streptococcus agalactiae* and *Streptococcus iniae* strains of different virulence following a 1 hour incubation in Nile tilapia blood. Values shown are mean percentage survival ± S.E.M. Means that do not share a letter are significantly different ($p < 0.05$).

3.3.5.1 Blood smears

Microscopic examination of blood smears showed better staining when the Rapid Romanowsky stain technique was used rather than the Giemsa staining protocol. There seemed to be little difference in the appearance of the blood smears between the two streptococcal species and between isolates. Free bacteria were visualised within the blood sample, the arrangement of the bacteria was individual, in chains or in clumps (Figure 3.7.A – B). Both *S. agalactiae* and *S. iniae* could be seen engulfed by macrophages (Figure 3.7.C – D) and there appeared to be marginally more lysed red blood cells in *S. iniae* samples compared with *S. agalactiae* (subjective visual observation) (Figure 3.7.H). It was difficult to ascertain whether bacteria were present within or surrounding the erythrocytes in the blood smear. As seen in Figure 3.7.E, F – G), what appears to be a bacterium could also be interpreted as micro-nucleated erythrocytes, vacuolated nucleus or nuclear retraction.

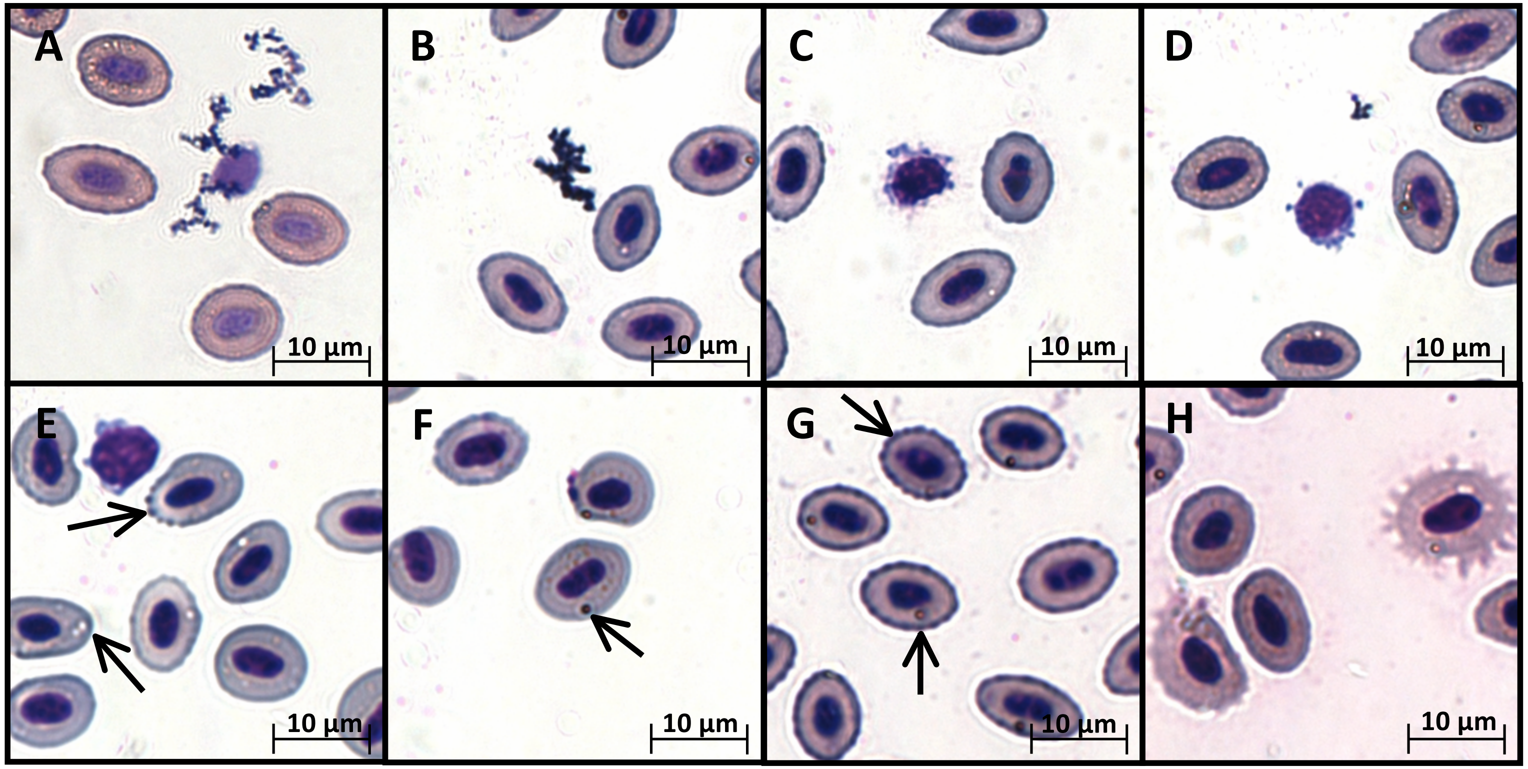

Figure 3.7 Blood smears from the blood survival assay stained with Rapid Romanowsky. [A] Free *S. agalactiae* A bacterium [B] Free *S. iniae* D bacterium [C] *S. agalactiae* C engulfed by a macrophage [D] *S. iniae* D engulfed by a macrophage [E – F] Blood smear of *S. agalactiae* D [G] Blood smear of *S. iniae* A [H] Burst red blood cells from *S. iniae* C blood smear. Arrows denotes areas of interest.

3.3.6 Haemolysin assay

A haemoglobin standard curve was measured at 5 different wavelengths on a spectrophotometer to determine the optimum wavelength to use in a haemolysin assay (Figure 3.8). The wavelength 450 nm was selected as this produced the highest absorbance readings and had a R^2 value of 0.9991. A positive haemolysin test was therefore defined as an OD_{450} reading that equalled or was above 0.33 (Figure 3.8).

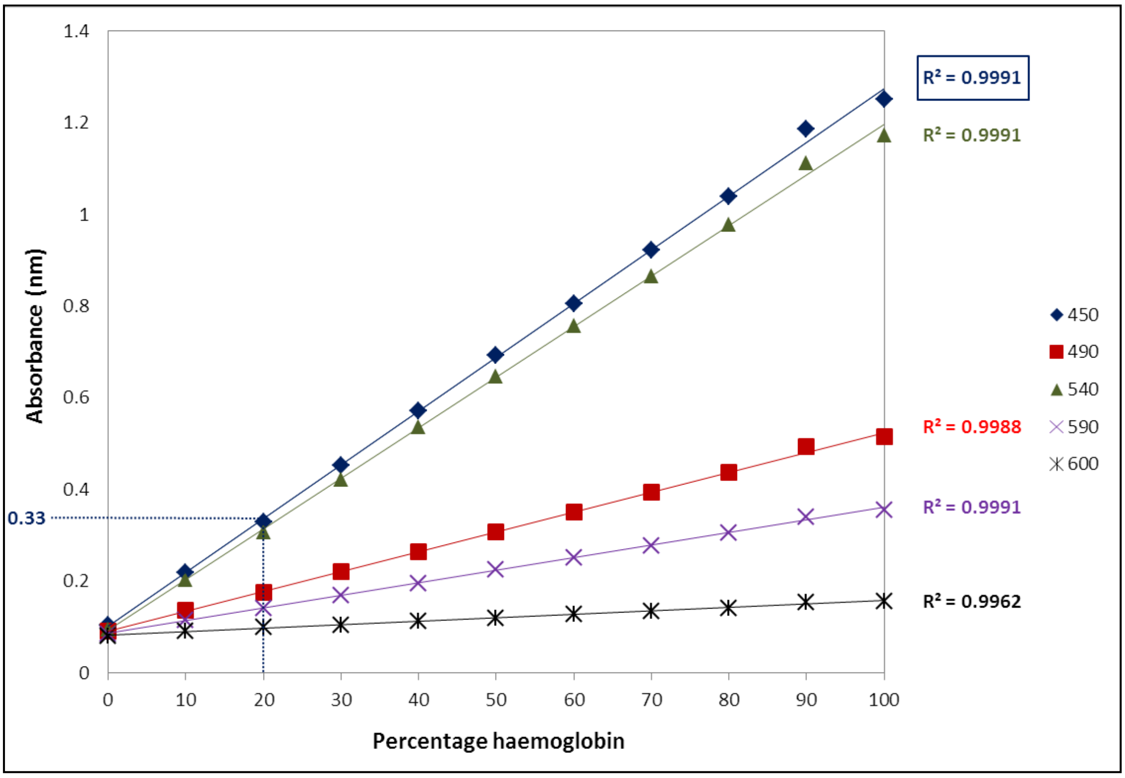

Figure 3.8 Haemolysin standard curve from sheep's blood measured at various absorbance wavelengths by spectrophotometry

Virulent *S. iniae* isolates had significantly higher haemolytic activity than the avirulent *S. iniae* strain and all of the *S. agalactiae* strains tested (p = 0.000) (Figure 3.9). The avirulent *S. agalactiae* B isolate was not significantly different to virulent *S. agalactiae* strains in its ability to lyse blood in this assay. There was no significant difference between replicates (p = 0.039).

The absorbance readings for all virulent *S. iniae* strains were above the positive haemolysin test value 0.33 (Figure 3.8). However, all other strains tested had absorbance readings below this value and therefore produced a negative result for this haemolysin test in accordance with the protocol from Rose and Okrend (1998).

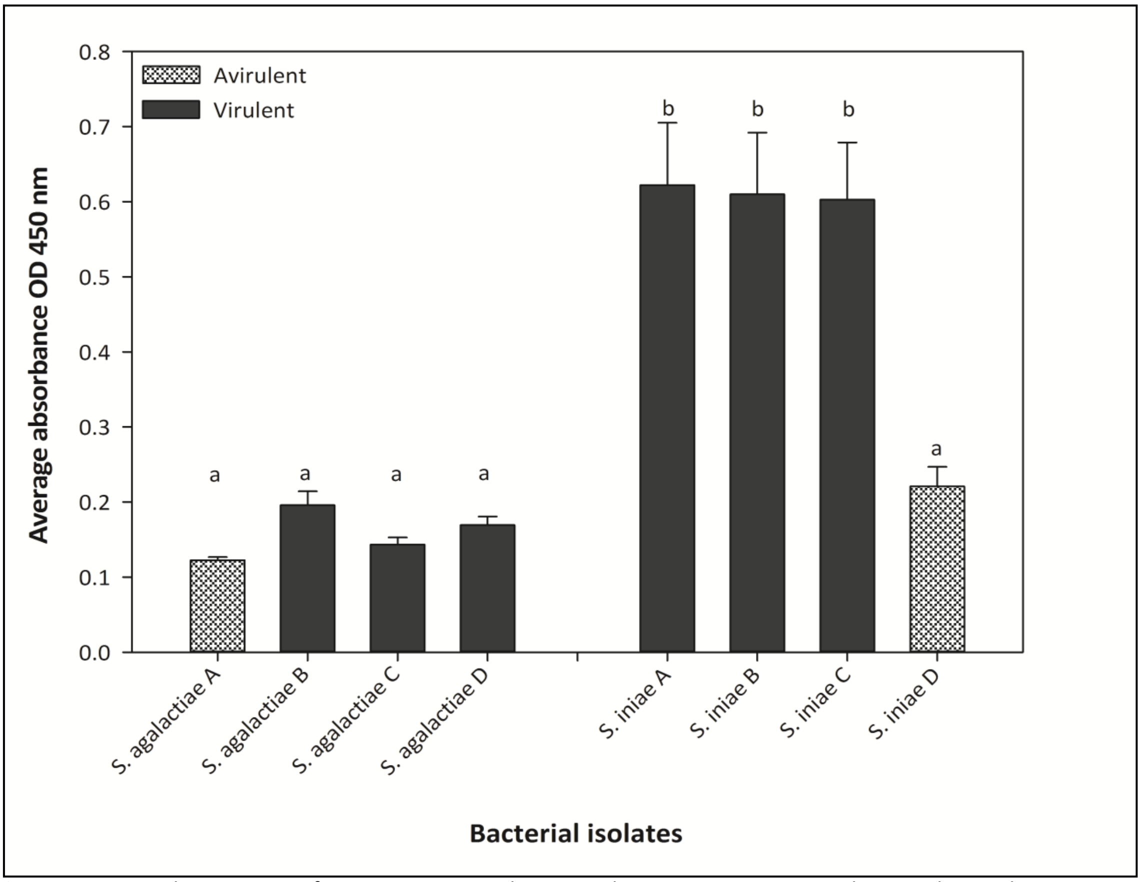

Figure 3.9 Haemolytic activity of *Streptococcus agalactiae* and *Streptococcus iniae* isolates. Values indicate mean haemoglobin release (± S.E.M.) from sheep's blood measured at 450 nm by spectrophotometry. Treatments that do not share a letter are significantly different ($p < 0.05$).

3.3.7 Complement-mediated killing assay

The mean percentage survival of *S. agalactiae* isolates after incubation in active and heat-inactivated Nile tilapia serum was similar. Consequently, the mean difference in percentage survival between these two groups was relatively small (Figure 3.10). The percentage survival of virulent *S. iniae* strains was greatly increased in heat-inactivated serum compared with active serum. The avirulent *S. iniae* isolate also had a higher percentage survival in heat-inactivated serum but the difference in survival between the two groups was not as substantial (Figure 3.10). The mean difference in percentage survival was significantly higher for *S. iniae* strains compared with *S. agalactiae* (p = 0.000). There was no significant difference between the eight different isolates (p = 0.063). There was a significant difference between replicates (p = 0.054), however the trend illustrated in Figure 3.10 was consistently seen in each replicate.

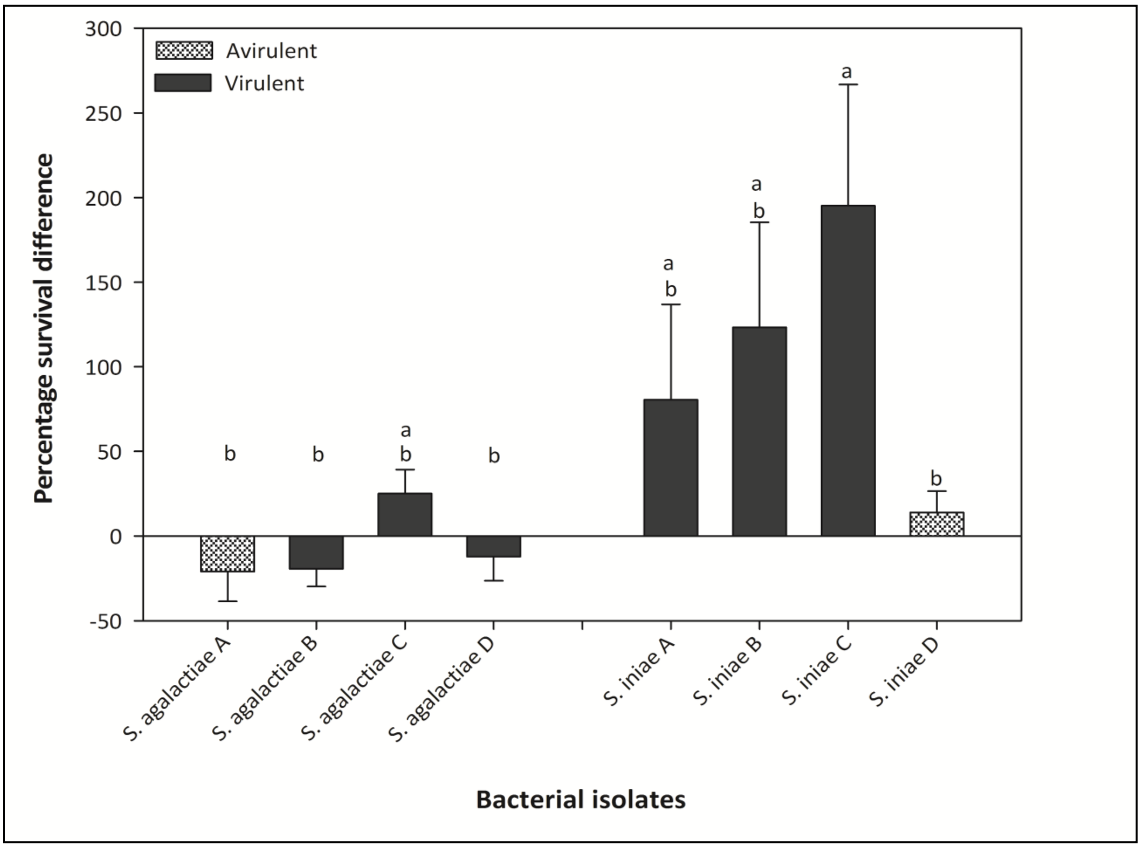

Figure 3.10 Effect of serum heat inactivation on bacterial survival calculated by subtracting average survival in active serum from average survival in heat inactivated serum. Values indicate the mean percentage survival difference ± S.E.M. Treatments that do not share a letter are significantly different ($p < 0.05$).

3.3.8 Determination of capsule presence

When the *Streptococcus* bacteria were examined using transmission electron microscopy no capsule formation was apparent on any of the samples. As seen in Figure 3.11 none of isolates exhibited an electron-dense layer on the cell surface.

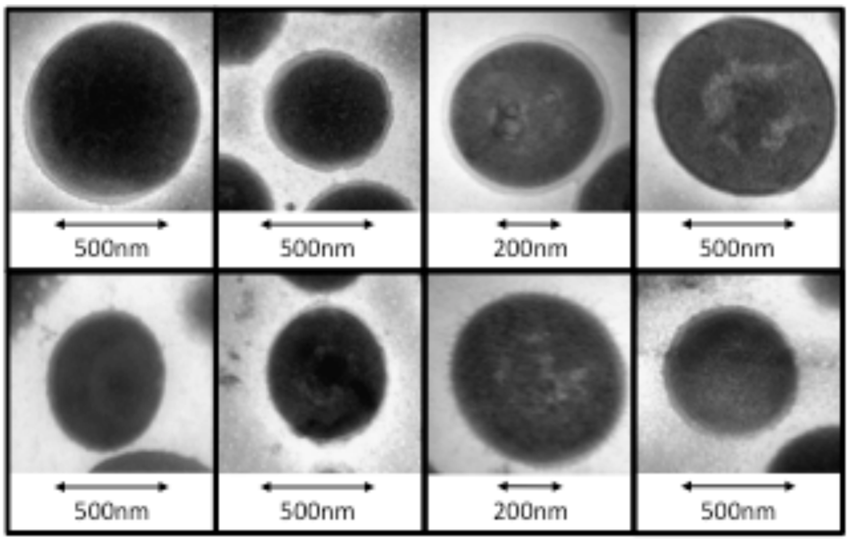

Figure 3.11 Transmission electron microscope images of *Streptococcus agalactiae* and *Streptococcus iniae*. Top row [left to right]: *S. agalactiae* A, B, C, D. Bottom row [left to right]: *S. iniae* A, B, C, D.

However, when Anthony's capsule stain method was utilised all *S. agalactiae* and *S. iniae* isolates tested showed the presence of a capsule when compared with the positive control. As seen In Figure 3.12 the bacterial cells and the proteinaceous background appear purple/blue with the capsule forming a transparent outline surrounding the bacterium.

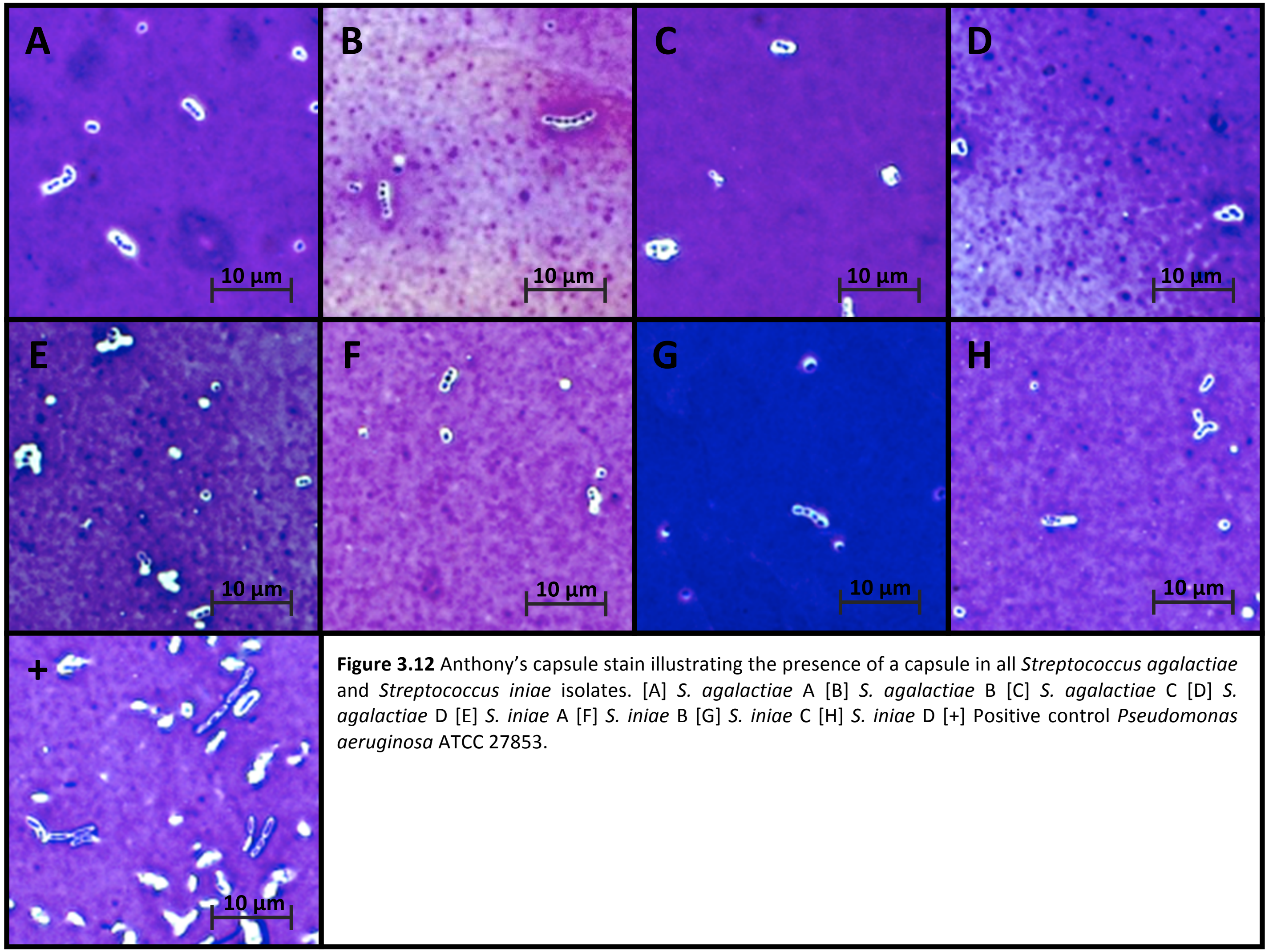

Figure 3.12 Anthony's capsule stain illustrating the presence of a capsule in all *Streptococcus agalactiae* and *Streptococcus iniae* isolates. [A] *S. agalactiae* A [B] *S. agalactiae* B [C] *S. agalactiae* C [D] *S. agalactiae* D [E] *S. iniae* A [F] *S. iniae* B [G] *S. iniae* C [H] *S. iniae* D [+] Positive control *Pseudomonas aeruginosa* ATCC 27853.

3.3.9 Virulence genes of *Streptococcus iniae*

All of the *S. iniae* isolates tested positive for the presence of virulence factor genes cytolysin SLS (*sagA*), M-Like protein (*simA*), C5α peptidase (*scpI*), capsule (*cpsD*) and polysaccharide deacetylase (*pdi*) as amplification products were present at the expected band sizes (Table 3.8). From the phosphoglucomutase (*pgm*) primers, amplified products were apparent for all *S. iniae* samples but not at the expected band size of 713 bp, instead band sizes at approximately 500 bp were found. No amplified products were found for the Milli-Q water or *S. agalactiae* NCIMB 701348 controls except for the *simA* primers were a band at approximately 700 – 800 bp was found for *S. agalactiae*.

Table 3.8 Results of several PCR assays for the detection of virulence factor genes in a range of aquatic *Streptococcus iniae* strains. When amplified products were present at the expected band size it was considered as a positive result and represented by a tick (✓).

		Virulence factor genes					
Bacterial species and strain		***simA* (994 bp)**	***pdi* (381)**	***scp* (872)**	***pgm* (713)**	***cps* (534)**	***saga* (190)**
S. iniae	A (B99115)	✓	✓	✓		✓	✓
	B (B99120 1103-99)	✓	✓	✓		✓	✓
	C (B99130 Kidney)	✓	✓	✓		✓	✓
	D (J39)	✓	✓	✓		✓	✓
	B99120 1090/99	✓	✓	✓		✓	✓
	B99120 1104/99	✓	✓	✓	× bands seen at ≈ 500 bp	✓	✓
	B99120 1105/99	✓	✓	✓		✓	✓
	B99120 1121/99	✓	✓	✓		✓	✓
	B99120 1121/99 P	✓	✓	✓		✓	✓
	B99130 B	✓	✓	✓		✓	✓
	ATCC 29178	✓	✓	✓		✓	✓
	ATCC 29178	✓	✓	✓		✓	✓
S. agalactiae NCIMB 701348		× band seen at ≈ 750 bp	×	×	×	×	×

3.4 Discussion

All of the bacterial isolates investigated in this study were originally recovered from natural infections in aquatic animals, mostly fish. However, little was known about their virulence capacity nor associated virulence factors, particularly when tested in tilapia. Different host species and storage conditions of the bacteria may influence the pathogenic ability of the organism to cause disease in tilapia. Consequently, it was not unexpected that some of the bacterial strains tested did not cause morbidity or mortality during the passage experiments. However, the variance in the virulence ability found between bacterial strains enabled assays to be performed that could investigate virulence factors. Through the direct comparison between virulent and avirulent strains an insight into the pathogenicity of infection each bacterial species employ could be ascertained.

By examining API 20 STREP results throughout the fish passage study, it was found that some of the bacterial strains were able to adapt to changes in their environmental surroundings and alter their metabolism accordingly. However, after a short time in storage, any changes that occurred in enzyme activity were no longer apparent. This suggests that either [a] enzyme expression is a short term-effect phenomenon but that it can be recovered after passage through fish [b] enzyme expression may not be required when bacteria are grown on a general-purpose medium or [c] enzyme expression only occurs *in vivo*. Results for raffinose-inducible α-galactosidase, cellobiose-inducible β-glucuronidase and lactose-inducible β-galactosidase were found to be negative for several bacterial strains directly after the bacterium were recovered from passage. A likely explanation for this involves the regulatory process called carbon catabolite repression (CCR). In order to maintain optimal bacterial growth, a bacterium may select particular energy sources to utilise systematically when faced with a variety of carbon and energy sources obtained from its host. Otherwise simultaneous metabolism of all free sugars would be inefficient and subsequently lead to diminished growth. Therefore, CRR may up or down regulate genes specific for sugar utilisation until they are required by the bacterium for metabolism (Iyer *et al*., 2005). The ability of the bacterium to monitor its environment and react accordingly may therefore aid the virulence ability of the bacterium, by assisting in long term survival and promoting growth. However, in this study the ability of the isolates to alter their expression of particular enzymes was not associated with virulence. Nevertheless, it has been made apparent that API 20 STREP results may differ depending on the storage of the bacterium used or if it had been sub-cultured. This therefore provides an additional explanation for the variation in API 20 STREP results reported in the literature for *S. agalactiae* and *S. iniae* (Table 2.3).

It is important to understand the growth kinetics of each bacterial isolate for appropriate interpretation of growth data and for optimisation of growth and product formation (Kun, 2013). Kun (2013) highlights that the production of growth curves helps determine when bacterial cultures should be harvested depending on the experimental design. For example, if a bacterial growth-linked product or a non-growth-linked product is required the bacterial culture should be harvested in the exponential growth phase or the stationary growth phase respectively. Furthermore some bacterial species, such as *S. pyogenes,* have been shown to sequentially express genes involved in various aspects of their physiology, metabolism and virulence in a growth phase-dependent manner (Chaussee *et al*., 2008; Sitkiewicz and Musser, 2009). Likewise, Sitkiewicz and Musser (2009) also found that *S. agalactiae* differentially expressed several genes throughout its growth. It is theorised that virulence genes in particular are up or down regulated in accordance with growth as this coincides with their mode of infection. For example, Sitkiewicz and Musser (2009) suggested that cell surface proteins may be produced during the early stages of infection, promoting adhesion to host cells, but later down regulated to evade detection by the host's immune system. This conjecture was supported by their results as it found that the production of virulence factors involved in the establishment of infection was reduced during growth. This incorporates an extra level of complexity when studying virulence factors through experimental assays; it is critical to ensure that bacterial suspensions utilised during procedures are of the same growth phase. Similarly, it must also be noted that if virulence factors are found lacking within particular environmental conditions, this does not mean that the bacterium cannot produce the virulence factor within another environment and/or growth phase. Results may therefore need to be used in conjunction with molecular work to ascertain if the bacterium contains the gene(s) required for production and regulation of the particular virulence factor.

Since virulence factor production may be growth-phase dependent, a bacterial culture that was in late log phase for both bacteria species was used in all assays in this study. This improved the chance that any virulence factor produced in either log or stationary phase may be observed in the assay. Both *S. iniae* C and *S. agalactiae* B demonstrated typical growth curves of a microbial batch culture by their distinct associated phases of growth; lag, growth, stationary and death phases. Under the tightly controlled *in vitro* conditions *S. iniae* C had a faster exponential growth phase than *S. agalactiae* B and subsequently reached stationary phase before *S. agalactiae* B. The exact time when *S. iniae* C entered stationary phase post-incubation was also more difficult to determine compared with *S. agalactiae*. This may be due to the higher growth rate of *S. iniae* resulting in the bacterium exhausting its nutrient source rapidly. This results in a short stationary phase as the culture medium is no longer able to support any remaining viable bacterial cells and thus prompting the rapid onset of the death phase.

Only one bacterial isolate was used to characterise the growth curve of each bacterial species. Previous research showed that different strains of *S. agalactiae* had similar growth curves (Wongsathein, 2012) therefore using additional strains was deemed unnecessary. Once a rough approximation of the growth phases was ascertained, the bacterial concentration of all *S. agalactiae* and *S. iniae* at the late log phase was established using viability counts. This was more time and cost effective than duplicating growth curves.

There is a variety of different techniques that can be used for bacterial enumeration such as: most probable number, 6x6 plate method, serial plate method and direct plating onto agar-based medium (pour, drop, spread and spiral plating) (Barbosa *et al*., 1995; Chen *et al*., 2003). Bacterial enumeration through the drop count method is widely established in microbiological research. There is, however, no standardised procedure for this technique (Chen *et al*., 2003; Herigstad *et al*., 2001). The drop count method has been suggested to be superior to other methods in estimating viable cells numbers as it has higher precision and is more time concise (Herigstad *et al*., 2001). However, it can be sensitive to operator error due to serial dilution and pipetting techniques. From this study, inconsistencies in enumerating cfu between sectors on the same TSA plate and between replicates were found. It was not within the scope of this study to evaluate the causes of such variations, however, Barbosa *et al*. (1995) found the counting of cfu from cluster forming bacteria led to more variable results than counting non-cluster forming bacteria. It is unknown whether these chain forming *Streptococcus* species are also cluster forming bacteria, however results did indicate that *S. agalactiae* and *S. iniae* were not evenly distributed throughout the suspension when samples were taken.

It has been suggested that the preferred countable dilution should produce 3 – 30 colonies per 10 µl drop of bacterial suspension (Herigstad *et al*., 2001). However, in this study it was found that this recommended range could not always be achieved, with concurrent dilution factors providing colonies counts below (< 3) or above this number (> 30). Consequently, it is necessary to employ an efficient methodology that acknowledges this drawback. Therefore, it has been recognised that during future experimental procedures enumerating cfu values should be represented as a range or estimation of bacterial numbers using multiple dilution factors when appropriate.

In contrast to human *S. agalactiae* strains, all aquatic *S. agalactiae* strains tested in this study were non-haemolytic. This was confirmed in both the haemolysin assay and examination of bacterial growth on SBA. This indicates that the lysis of red blood cells may not be required for aquatic *S. agalactiae* pathogenicity. On the other hand, virulent *S. iniae* strains all showed haemolytic abilities in the haemolysin assay whereas the avirulent *S. iniae* strain (*S. iniae* D) did not. The loss of virulence of *S. iniae* D may therefore be attributed to decreased haemolytic activity. However, when *S. iniae* D was inoculated on SBA, zones of haemolysis were apparent after a 2-day incubation period. This would suggest that *S. iniae* D has the ability to break down blood but at a slower rate than the virulent strains and it is this somewhat delayed haemolysis that may have contributed towards a loss of virulence.

Previous studies have investigated *S. iniae* resistance to whole blood killing with varying outcomes. After incubation in blood from hybrid striped bass (*Morone chrysops* × *M. saxatilis*) some *S. iniae* strains showed a 400% survival rate from the initial bacterial cfu count whereas other strains only showed an 80 – 120% survival rate (Buchanan *et al*., 2008; Locke *et al*., 2007). The impact of strain variation has also been shown by Fuller *et al*. (2001) where different *S. iniae* strains incubated in human blood had a percentage survival ranging from 0.4 – 77%. The blood types used in such assays (Zlotkin *et al.*, 2003) and the pathogenic ability of the bacterium (Buchanan *et al.*, 2008) proved to significantly affect the ability of *S. iniae* strains to survive in blood. Due to the differences in the blood type, incubation temperature and incubation time used in other studies, making constructive comparisons with this current study is challenging.

Nevertheless, current findings indicate that the virulence status of either species does not affect the bacterium's ability to survive in blood. However, a significant difference was observed between *S. agalactiae* and *S. iniae* strains, which indicates that these two bacterial species may employ different modes of infection.

As highlighted by Buchanan *et al*. (2008), whole blood killing consists of the combined antibacterial activities of serum and circulating phagocytic cells (neutrophils and macrophages). Therefore, the higher percentage survival of *S. agalactiae* strains indicates that this species has better resistance to complement-mediated cell lysis and phagocytic clearance. Findings from the complement-mediated assay presented in this study supports this; there were relatively minor differences in the percentage survival of *S. agalactiae* strains incubated in active and heat-inactivated serum. While virulent strains of *S. iniae* showed considerably higher survival in heat-active serum indicating their sensitivity to serum complement. However, this may actually be an adept strategy by *S. iniae* and a key stage in the pathogenesis process. Zlotkin *et al.* (2003) suggest that *S. iniae* have an '*in vivo* intracellular lifestyle' as it was found that some *S. iniae* strains have the capability of entering into and multiplying within macrophages prior to macrophage apoptosis. Zlotkin *et al.* (2003) subsequently proposed that apoptotic phagocytes serve as vectors that are loaded in the blood circulation and are unloaded in the central nervous system after crossing the blood-brain barrier. The avirulent *S. iniae* strain had a similar percentage survival rate in active and heat-inactive serum indicating that it could not exploit the same intramacrophage lifestyle and thus mode of infection compared to its virulent counterparts. There was a significant difference between replicates for this assay but all the replicates showed a similar pattern of results. This may have been due to incomplete inactivation of the blood serum.

Surface capsular polysaccharides have previously been shown to be an important virulence factor for several *Streptococcus* species. The principle role of the capsule is to lower the rate of phagocytosis which is employed by the host's immune system to clear and eliminate foreign pathogens (Lowe *et al*., 2007). Previously research has shown that *S. iniae* fish isolates possess a capsule (Barnes *et al.,* 2003) and transposon mutagenesis has shown that the polysaccharide possesses a function in virulence (Shutou *et al*., 2007).

Although the presence of a capsule is noted in literature as an essential virulence factor for *S. iniae* in establishing an infection, there are reports of non-capsulated virulent strains (Fuller *et al.*, 2001). Kanai *et al*. (2006) characterised two serological phenotypes in Japanese *S. iniae* isolates by the presence or absence of a capsule, identified as K^+ and K^- respectively. Their research indicated that although the non-capsulated strains were derived from diseased fish they proved to be avirulent in a challenge model. Kanai *et al*. (2006) theorised that K^- strains could have derived from K^+ strains but have been transformed in the host; therefore the K^- strains may have been isolated from fish recovering from streptococcosis. There is evidence to support the theory that capsule production is actually regulated during an infection in accordance with different environments and/or tissue types within its host. Lowe *et al*. (2007) found that either reduced or excess capsule expression for *S. iniae* was advantageous in some environments but detrimental in others.

Vaccination programs have also been shown to prompt, by either driving mutation or natural selection, the formation of non-capsulated and novel capsular *S. iniae* serotypes which are still able to infect the host (Eyngor *et* al., 2008; Millard *et al*., 2012). Non-capsulated strains were found to produce lower mortality rates than capsulated strains as they are more susceptible to phagocytic attack. However, it is speculated that these non-capsulated *S. iniae* strains may modify their mode of infection and seek refuge in the bone of already-compromised fish therefore promoting their continued survival (Millard *et al*., 2012).

Ten capsular serotype types (Ia, Ib or II-IX) of *S. agalactiae* have been identified and associated with human infection (Cieslewicz *et al*., 2005; Rajagopal, 2009). Such variation in capsular structures has also been found in *S. pneumoniae*, where over 90 distinct capsular types are known (Cieslewicz *et al*., 2005). These serotypes are thought to be a result of selective pressure imposed by the host's immune response (Cieslewicz *et al*., 2005). Regardless of the serotype, the polysaccharide capsule is deemed a major virulence factor in human *S. agalactiae* infection (Sellin *et al*., 2000) through its role in resisting complement-mediated opsonophagoctic killing by blood leukocytes (Cieslewicz *et al*., 2005). The antiphagocytic properties of the sialic acid-rich capsular polysaccharide of *S. agalactiae* are thought to arise as a result of preventing complement factor C3 deposition on the bacterial surface (Lowe *et al.,* 2007; Marques *et al*., 1992). This inhibits activation of the alternative pathway of complement and thus interrupts neutrophil opsonophagocytic killing mechanisms (Lowe *et al*., 2007).

Similar to *S. iniae*, *S. agalactiae* is thought to regulate its capsule expression in response to the host and/or the external environment (Rajagopal, 2009). The vast majority of research investigating *S. agalactiae* capsules is based on human strains. However, Delannoy *et al.* (2013) found that fish *S. agalactiae* strains were either serotype Ia, Ib or III and work from Rosinski-Chupin *et al*. (2013) showed that fish strains possessed the 16 genes involved in the type Ib capsule synthesis.

Capsular biosynthesis in *S. iniae* is under the control of a 21-kb operon containing around 20 genes. However, variation in capsular genotype is limited to only a few genes within this operon, namely: *cpsY, cpsD, cpsE, cpsG* and *cpsH* (Millard *et al*., 2012). The *cpsD* gene which is required for capsule polymerisation and export in *S. agalactiae and S. pneumoniae* has also shown to be required for complete *S. iniae* capsule expression (Locke *et al*., 2007).

In this study it was important to investigate the presence or absence of a capsule to help determine the mode of infection *S. agalactiae* and *S. iniae* strains may employ. All *S. iniae* isolates used in this study possessed the *cpsD* gene required for capsule expression but a capsule was not observed using transmission electron microscopy in either *Streptococcus* species. The likely explanation is the protocol used did not preserve the capsule; however, the absence of a positive control in this assay makes this difficult to confirm. Previous studies that have successfully visualised the capsule using TEM have used either a lysine acetate fixation protocol (Locke *et al*., 2007), polycationic ferritin and antibody technique (Barnes *et al*., 2003) or lysine-based aldehyde-ruthenium red fixation protocol (Hammerschmidt *et al*., 2005). Anthony's capsule stain method was investigated in this study as a cheaper, quicker and alternative method to detect capsule presence. Although this method did not provide detailed information regarding the capsule, i.e. capsule size/amount expressed, it was able to illustrate the presence of a capsule for all bacterial strains investigated. These results illustrate that there is no variation in capsule presence between different strains or species tested, regardless of their virulence. Consequently, this implies that the inability of the avirulent strains to cause morbidity and/or mortality was not due to a loss in capsule expression.

Results from this study are in agreement with finding with Baums *et al*. (2013) whereby *S. iniae* strains have the genes for the virulence factors cytolysin SLS, M-Like protein, C5α peptidase, capsule and polysaccharide deacetylase. The function of these virulence factors has been discussed previously (Table 1.8). Due to the variety of *S. iniae* strains utilised in this study these findings imply that these genes may be universally present in *S. iniae* strains, however the sample number was small (n = 11) so confirmation of this would require the examination of considerably more strains. The presence of a virulence gene does not always equate to activation and expression of the virulence factor. To fully comprehend the activation and expression of virulence factors an assessment into genes coding for the particular virulence factor should be assessed and complemented with assays to determine virulence factor expression. As shown with capsule expression, one of the associated virulence genes, *cpsD*, was present in *S. iniae* strains but expression was determined through the Anthony's capsule stain. This process should be extended to the remaining virulence genes tested to ascertain if the related virulence factors are indeed expressed. However, this may prove challenging as the common method of assessing virulence factors is through allelic exchange mutagenesis therefore assays assessing actual virulence factor expression is somewhat limiting.

Genes coding for *S. agalactiae* virulence factors were not investigated in this study. A study by Delannoy *et al*. (2013) has already examined the presence of a small number of virulence genes in the fish isolates used in this study. From Delannoy's work it would appear that *S. agalactiae* A, B, C and D do not have the virulence gene *rib* which is associated with the resistance to protease immunity protein. However, all these strains do possess the gene *bca* which encodes for Cα protein. In addition, *S. agalactiae* A, B and D were shown to contain the *bac* virulence gene which is associated with the virulence factor Cβ protein whereas *S. agalactiae* C did not contain this gene. Consequently, strains of aquatic *S. agalactiae* can differ in the virulence gene they contain and resultantly the virulence factor they may express. Again, the function of these virulence factors has been discussed previously (Table 1.7). The vast majority of research on *S. agalactiae* virulence factors is founded on human strains. To extend such research and incorporate fish isolates would therefore require the use of *S. agalactiae* human strains to act as controls which were not available at the time of this study.

In summary, the results from this study demonstrated that the aquatic *S. agalactiae* and *S. iniae* tested possessed the same virulence factors such as the possession of a capsule but shown to differ in their ability to break down blood, survive in blood and resist complement-mediated killing. Through these results it is theorised that the virulent aquatic *S. agalactiae* strains may initially have a more systemic spread of infection. Whereas virulent aquatic *S. iniae* strains may utilise a more localised spread of infection within the host.

3.5 References

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Chapter 4

Evaluating streptococcal interactions: competition or coexistence

4.1 Introduction

Outbreaks of streptococcosis have been reported in many countries around the world, in a wide variety of fish species all farmed under varied environmental conditions. Although a disease outbreak of streptococcosis is usually attributed to a single pathogen both *Streptococcus agalactiae* and *S. iniae* have been found present in the same geographical locations (Conroy, 2009) and on the same farm during a disease outbreak (M. Crumlish, personal communication, 2010). Independent infection models performed under experimental conditions have been developed for *S. iniae* and *S. agalactiae* in Nile tilapia (*Oreochromis niloticus*) (Baums *et al*., 2013; Wongsathein, 2012). The reasons for the experimental challenge studies are numerous but such studies traditionally involve exposure of the fish via varied routes to a single pathogen. However, intensive aquaculture systems will harbour a wide diversity of microorganisms including multiple potential pathogens. Consequently, it is unlikely that fish will encounter only one disease causing agent at a time and concurrent infections are more representative of the host-bacterial interactions within a farming environment.

The standard code of practice in the UK underpinning the use of animals in experimental scientific research is centred on the three Rs: replacement, refinement and reduction. Replacement refers to methods that avoid or replace the use of animals defined as 'protected' under the Animals (Scientific Procedures) Act 1986, amended 2012 (ASPA, 2012). Protected animals include any living vertebrate (other than man), cephalopods and *Octopus* *vulgaris*. Protection extends to immature forms: [a] mammals, birds and reptiles – from halfway through the gestation or incubation period and [b] fish, amphibian and *O. vulgaris* – from the time at which they become capable of independent feeding (ASPA, 2012). Alternative host models using 'lower species' such as insects have been widely researched in an attempt to replace these 'protected' animals in experimental designs. Therefore prior to conducting any large *in vivo* fish experimental work to establish a co-infection a study was performed to investigate the effects of a simultaneous bacterial infection using the wax moth larvae (*Galleria mellonella*). This was in direct compliance with the UK Home Office NC3rs.

Whilst there are numerous alternative animal experimental models proposed, very few of them are applicable to fish pathogens. However, the wax moth larvae have been successfully used in the study of pathogenic microorganisms as an alternative to vertebrate experimental models (Evans and Rozen, 2012; Olsen *et al*., 2011). There are several advantages of using wax moth larvae for infection models including practicality, ethics and costs. Wax moth larvae are widely available and at a low cost (typically £1.50 per 40 – 50 larvae), no specialist equipment is required to house the larvae and very little training is required to inoculate them (Desbois and Coote, 2012). Importantly, they are considered as an ethically acceptable alternative animal infection model (Desbois and Coote, 2012; Peleg *et al*., 2009).

The wax moth larvae have been used in infection models for numerous pathogens including Gram-negative bacteria (Peleg *et al*., 2009), Gram-positive bacteria (Evans and Rozen, 2012; Olsen *et al*., 2011) and fungi (Brennan *et al*., 2002). However, as highlighted by Evans and Rozen (2012), due to differences in pathogenicity and mechanisms of infection between bacterial species the suitability of wax moth larvae must be tested for each pathogen. In this study an initial investigation examined the suitability of using wax moth larvae as an alternative animal model for exploring *S. agalactiae* and *S. iniae* infections during single pathogen and co-infection exposure in Nile tilapia (*Oreochromis niloticus*).

The role of microbial co-infections in disease outbreaks is not well understood for a wide range of diseases, but especially in aquaculture. As stated previously, due to the aquatic environment the animals are likely to be exposed to more than one organism, hence it might be that in aquatic farming co-infections, whilst complicated, are likely to contribute towards morbidity and mortality during a disease episode. Microbial interactions are complex and often involve cell signalling pathways controlled by varied factors including the number of bacteria, which may be influenced by environmental growth conditions and access to appropriate resources. If there are other potential pathogens in the same host that are both equally able to establish and cause disease, does this result in synergy, microbial competition or inhibition of one of the pathogens? These interactions may have significant consequences on the incidence and prevalence of a disease outbreak within a farming system, not to mention difficulty when implementing disease control and treatment strategies. Therefore, a study was performed to determine [a] suitability of wax moth larvae as a model host for the study of *S. agalactiae* and *S. iniae* infection and [b] to determine if there was any recognisable interaction between *S. agalactiae* and *S. iniae* as measured *in vitro*.

4.2 Materials and methods

4.2.1 *Galleria mellonella* infection model: Insect larvae

Wax moth larvae (*Galleria mellonella*) at the sixth development stage were obtained from Livefood UK, Somerset, England. Larvae were allowed to equilibrate for at least 24 hours by being stored in the dark at 4 °C with wood shavings and were used within 7 days. Only larvae with a cream coloured cuticle and minimal discolouration were used. Twenty randomly chosen larvae were used per treatment group for each experiment.

4.2.1.1 *Galleria mellonella* infection model: Preparation of inoculum

Four strains of *Streptococcus, S. agalactiae* A, *S. agalactiae* B, *S. iniae* C and *S. iniae* D (Table 3.1), previously passaged in fish, were grown on tryptone soya agar (TSA) (Oxoid Ltd, Basingstoke, UK) as previously described in Section 2.2.1. One colony from each pure culture was inoculated and grown in tryptone soya broth (TSB) (Oxoid Ltd, Basingstoke, UK) as previously described in Section 3.2.7. After incubation, samples were centrifuged at 2602 g for 15 minutes (MSE Mistral 2000R, MSE, London, UK).and the supernatant was discarded. The bacterial pellet was resuspended in phosphate buffered saline (PBS) (See appendix) and the optical density (OD) of each sample adjusted to give OD_{600} 1 measured at absorbance 600 nm using a WPA CO 8000 Cell Density Meter (Biochrom Ltd., Cambridge, UK). Further dilutions were then made for inoculation; 10^2, 10^4 and 10^6 cfu/10 µl. Bacterial concentration was confirmed by a viable cell count as previously described (Section 3.2.4).

4.2.1.2 *Galleria mellonella* infection model: Determining strain virulence

Separate groups of larvae were inoculated with three different bacterial concentrations (10^2, 10^4 and 10^6 cfu/10 µl) of either *S. agalactiae* A, *S. agalactiae* B, *S. iniae* C or *S. iniae* D. Larvae were injected with 10 µl of each bacterial suspension into the haemocoel through the last left pro-leg using a Hamilton syringe fitted with a 50 gauge needle. Larvae were held on ice during the inoculation process. Two types of controls were employed for each assay, these included larvae which were neither handled nor inoculated and larvae which were injected with PBS only. After inoculation, all larvae were kept in 90 mm petri dishes without wood shavings and incubated at 28 °C. Mortality rates were monitored every 24 hours for 7 days and determined through a lack of response to a physical stimulus. Each individual bacterial challenge used different larvae batches. Data was plotted using the Kaplan-Meier method and analysed using log rank tests. A value of $p > 0.05$ was considered significant and Holm's correction was applied to account for multiple comparisons.

4.2.1.3 *Galleria mellonella* infection model: Simultaneous inoculation

Triplicate groups of larvae were inoculated with a range of concentrations of *S. agalactiae* B (10^4, 10^5 and 10^6 cfu/10 µl) or *S. iniae* C (10^2, 10^4 and 10^6 cfu/10 µl). Concurrently, 9 larval groups were injected with a combined inoculum containing both *S. agalactiae* B and *S. iniae* C, each group had different concentrations of *S. agalactiae* and *S. iniae*. Larvae were randomly allocated to treatment groups from the 4 batches purchased. Data was analysed using ANOVA followed by Tukey *post-hoc* tests.

4.2.2 Bacterial competition as determined by cross plate and competing drop assays

Enterococcus faecalis NCIMB 775 and *Lactococcus garvieae* NCIMC 702155 were obtained from cryo-bead culture collections stored at -70 °C at the Institute of Aquaculture, University of Stirling. Passaged isolates of *S. agalactiae* A, *S. agalactiae* B, *S. iniae* C, *S. iniae* D (Table 3.1) were also sourced from a cryo-bead collection. Bacteria were revived as previously described (Section 2.2.1).

4.2.2.1 Cross-plate assay

The potential of *S. agalactiae* and *S. iniae* in inhibiting the growth of other bacterial isolates was assessed using cross-streak assays and competing drop assays. Eight cross-plate competition assays were performed. The bacterial strains that were used are shown in Table 4.1.

Table 4.1 Bacterial strains used in 8 cross-plate competition assays

Plate number	Primary bacterial species	Secondary bacterial species
1	*S. iniae* C	*S. iniae* D
2	*S. iniae* C	*S. agalactiae* B
3	*S. iniae* C	*E. faecalis*
4	*S. iniae* C	*L. garvieae*
5	*S. agalactiae* B	*S. agalactiae* A
6	*S. agalactiae* B	*E. faecalis*
7	*S. agalactiae* B	*L. garvieae*
8	*S. agalactiae* B	*S. iniae* D

One colony of the primary bacterial species was streaked six times onto half-strength TSA; the secondary bacterial species was streaked six times at a 90° angle to the primary bacterial species (Figure 4.1). The process of streaking the bacteria across the plate resulted in a reduction in the bacterial density from the start to the end in the line. This produced a matrix of overlapping lines at different bacterial concentrations. Plates were subsequently incubated for 2 days at 28 °C before bacterial growth was inspected.

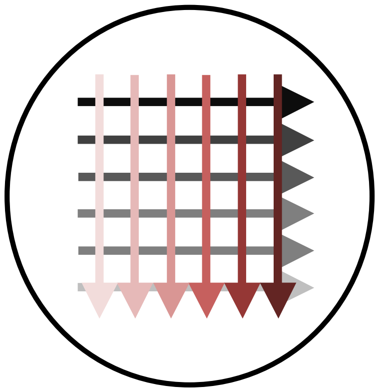

Figure 4.1 A diagram illustrating a cross-plate competition assay. One colony of the primary bacterial species (shades of black) was used to make six concurrent steaks of bacterial inoculum. The secondary bacterial species (shades of red) was streaked onto the TSA plate in the same manner at 90° perpendicular to the primary bacterial species.

4.2.2.2 Competing drop colonies

The protocol was modified from Kreth *et al.* (2005). The bacterial strains used and their arrangement for the 4 competing drop colony assays are shown in Table 4.2. A bacterial suspension was prepared in 0.85% saline as described in Section 3.2.7. Subsequently, a 10 µl bacterial suspension drop consisting of approximately 1 x 10^6 cfu was inoculated onto half-strength TSA plates as the early colonisers. Plates were left to dry for 10 minutes at room temperature before being incubated overnight at 28 °C. After incubation, 10 µl of the competing bacterial species, prepared as described above, was inoculated beside the early coloniser as the later coloniser. The two drop colonies were positioned so there would be an approximate 0 – 1 mm overlap between them. This experiment was repeated but inoculation of all species was made simultaneously beside each other. Plates were incubated for 2 days at 28 °C before bacteria growth was inspected.

Table 4.2 Bacterial strains and their arrangement for 4 competing drop colony assays

Plate number	Early coloniser	Late coloniser
1	*S. iniae* C	*S. iniae* D
	S. iniae C	*S. agalactiae* C
	S. iniae C	*S. agalactiae* D
	S. iniae C	*E. faecalis*
	S. iniae C	*L. garnieae*
2	Same as plate 1 but all inoculations made simultaneously	
3	*S. agalactiae* B	*S. iniae* D
	S. agalactiae B	*S. iniae* C
	S. agalactiae B	*S. agalactiae* D
	S. agalactiae B	*E. faecalis*
	S. agalactiae B	*L. garnieae*
4	Same as plate 3 but all inoculations made simultaneously	

4.2.3 Evaluation of bactericidal activity from *Streptococcus agalactiae* and *Streptococcus iniae in vitro*

To assess if either *S. agalactiae* or *S. iniae* has any bactericidal ability *in vitro* viable cell counts were performed on bacterial suspensions that were incubated with and without another bacterteria's supernatant being present. A simple schematic illustrating the methodology for this portion of the study is shown in Figure 4.3.

4.2.3.1 Determination of viable cell counts

(a) Determination of viable counts of *S. agalactiae* and *S. iniae* after treatment with filtrated supernatant.

One colony of *S. agalactiae* B and *S. iniae* C was inoculated into 20 ml TSB and incubated at 28 °C, 140 rpm for 10 hours then centrifuged at 2602 g for 15 minutes. The supernatant was collected and filtrated using a Millix-GP 0.22 µm syringe filter unit (Millipore, Massachusetts, USA). The bacterial pellet was resuspended in 0.85% saline to an OD_{600} 1 before a bacterial suspension containing approximately 50 cfu in 200 µl was made. The bacterial suspension was mixed with a range of amounts (0 – 200 µl) of *S. iniae* and *S. agalactiae* filtered supernatant. The mixtures were then incubated at 28 °C, 140 rpm for 2 hours before a 100 µl sample was spread onto a TSA plate and incubated at 28 °C for 48 hours. The number of cfu was determined using a Stuart cell counter (Bibby Scientific Ltd, Stone, UK). Bacterial suspensions were also incubated with 200 µl TSB only to act as a control. To determine the number of cfu in the initial bacterial suspension 100 µl of each bacterial suspension was spread onto a TSA plate prior to the 2 hour incubation. All work was repeated twice.

(b) Filtered supernatant treated with proteinase k

This was performed as described above (a) with the exceptions that both bacterial supernatants were treated with proteinase K (Bioline, London, UK) before being incubated with bacterial suspensions. Two millilitres of each filtrated supernatant was treated with 20 µl of 10 mg/ml proteinase K and heated at 56 °C for 1 hour on an Eppendorf Thermomixer Comfort (Eppendorf, Stevenage, UK). The enzyme was then inactivated, using the same Thermomixer, by heating at 95 °C for 10 minutes.

(c) Filtered supernatant re-filtered with centrifugal filter units

This was performed as described previously (a) with a few exceptions: (1) the bacteria and supernatant mixture was incubated for 3 hours and (2) 4 ml of both bacterial supernatants were re-filtered using centrifugal filter units prior to incubation with bacterial suspensions. Bacterial supernatants were placed into Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-100 membrane and Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-10 membrane (Millipore, Massachusetts, USA) and centrifuged according to manufacturer's instructions. This produced 4 separate supernatant solutions for each bacterial suspensions [1] concentrated supernatant containing molecules < 10 kDa [2] supernatant containing molecules > 10 kDa [3] concentrated supernatant containing molecules < 100 kDa and [4] supernatant containing molecules > 100 kDa (Figure 4.2). The concentrated solutes [1 and 3] were resuspended into TSB to make a total volume of 4 ml. This assay was repeated with the incorporation of the effluent supernatant that remained in the centrifuge tube after filtration (Figure 4.2).

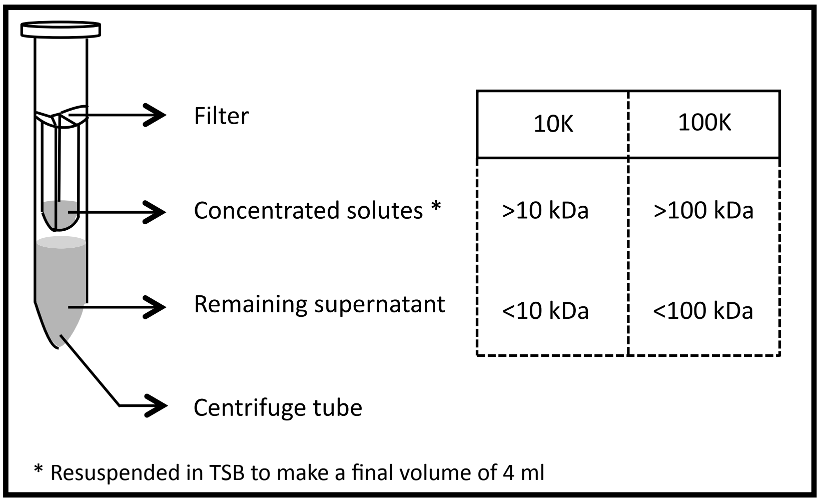

Figure 4.2 Amicon Ultra-4 centrifugal filter device used for the recovery and concentration of any extracellular products in *Streptococcus agalactiae* and *Streptococcus iniae* supernatants.

4.2.3.2 Bacterial protein expression measured by SDS-PAGE

The protocol for the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was based on that from Laemmli (1970) with modifications. The 4 separate supernatant solutions produced from Section 4.2.3.1(c) were used for this assay with the exception that the concentrated solutes were not resuspended in TSB. The supernatant solutions were run on an SDS-PAGE to separate any macromolecules according to their electrophoretic mobility. Fifty microliters of each sample was diluted in 50 µl of 2 x sample buffer (See appendix) and boiled for 2 minutes. After the samples were cooled to room temperature, 10 µl was dispensed onto two 12.5% acrylamide gels (See appendix) with 1 x running buffer (Pro-Pure x 20 Running buffer, Amresco, Solon, USA). Ten microlitres of Spectra multicolour broad range protein ladder (Thermo Fisher Scientific Inc, Wilmington, USA) was also dispensed onto the gel. Electrophoresis was carried out at 150 V, 313 mA, 100 W (Hoefer SE250 mini-vertical gel electrophoresis unit, Hoefer, Holliston, USA) for 1.5 hours or until the dye-front was approximately 1 cm from the bottom of the gel. Gels where then either stained with Coomassie brilliant blue or silver-staining to allow the separate protein bands to be visualised. The approximate molecular mass of each protein could then be estimated using the Spectra protein ladder. An image of both gels was taken using a light box and Nikon D300s camera with an 18 – 55 mm F4.5 lens.

(a) Coomassie brilliant blue staining

One SDS-PAGE gel was submerged in Coomassie Brilliant Blue R-250 solution (Fisher Scientific, Loughborough, UK) (See appendix) and left to develop for 18 hours with shaking (15 rpm) (Stuart Scientific Gyro-rocker, Bibby Sterlin Ltd., Stone, UK). The gel was submerged in destaining solution (See appendix) for 1 hour at 15 rpm then fresh de-stain was used for another 1.5 hours at 15 rpm.

(b) Silver-staining

The remaining SDS-PAGE gel was stained using ProteoSilver Silver stain kit (Sigma-Aldrich, Buchs, Switzerland) according to manufacturer’s instructions.

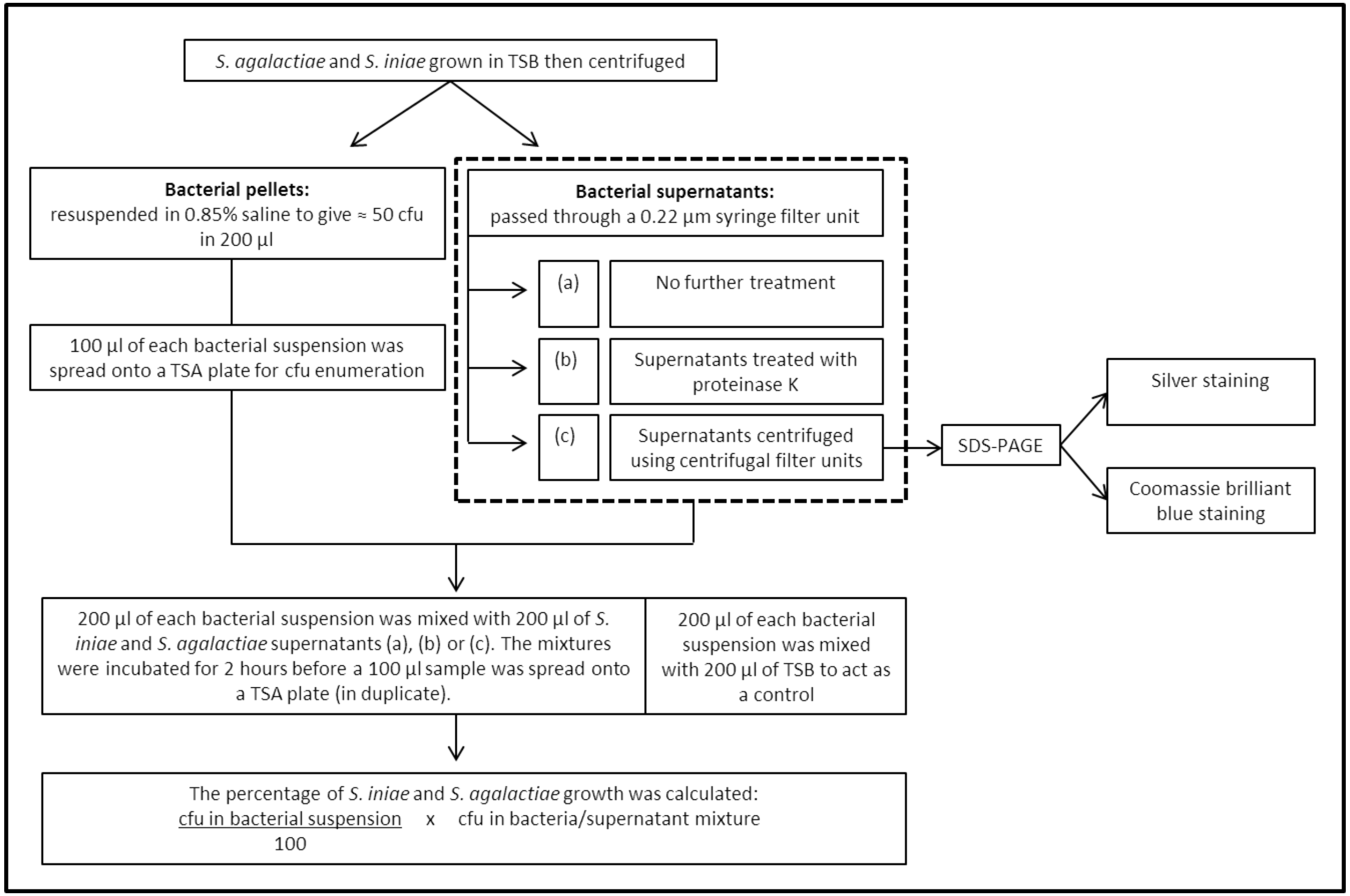

Figure 4.3 A schematic illustrating the methodology for the investigation into *Streptococcus agalactiae* and *Streptococcus iniae* supernatant.

4.3 Results

4.3.1 Determining the virulence of *Streptococcus* strains using wax moth larvae

Both *S. agalactiae* A (avirulent in tilapia) and *S. agalactiae* B (virulent in tilapia) killed larvae in a dose dependent response (Figure 4.4). This suggested that the larval model for *S. agalactiae* did not correlate with the *in vivo* virulence testing performed in fish.

Streptococcus iniae D, which was considered to be avirulent in tilapia, also appeared to have no effect when injected into the wax moth larvae. A small number of mortalities were observed (0 – 10%), however these were not dose related (Figure 4.5). *Streptococcus iniae* C, which was found to be virulent when tested in tilapia, caused a dose dependent reduction in larval survival (Figure 4.5). No mortalities ocurred in the control larval groups.

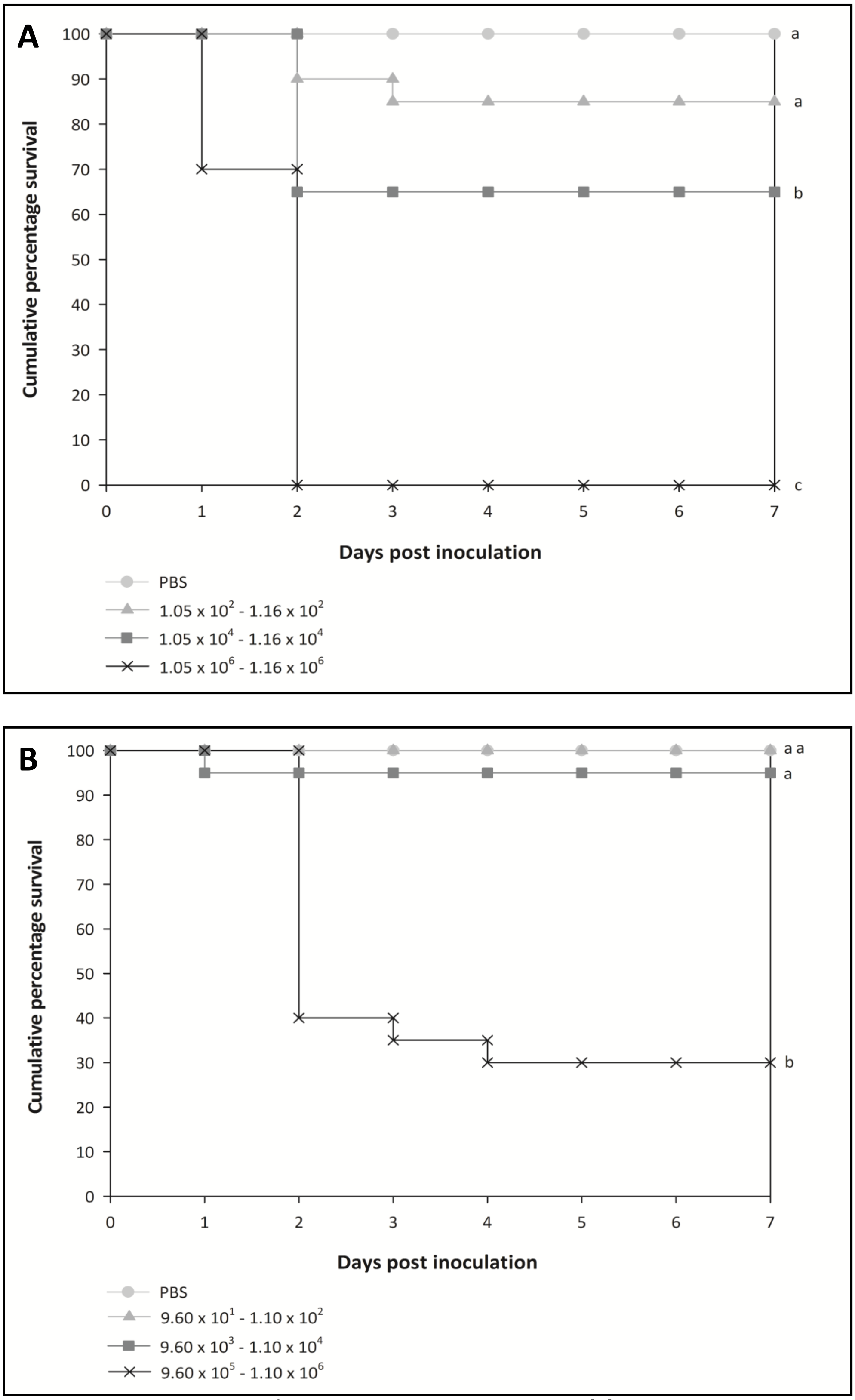

Figure 4.4 Kaplan-Meier survival curve for wax moth larvae inoculated with [A] *Streptococcus agalactiae* A and [B] *Streptococcus agalactiae* B at a range of concentrations over a 7 day period. Bacterial concentrations represent the concentration of bacterium per inoculum per larvae (cfu/10 µl). The bacterial concentration is presented as a range based on the viable cell count results. Treatments that do not share a letter are significantly different. A value of $p < 0.05$ was considered significant and Holm's correction was applied to account for multiple comparisons.

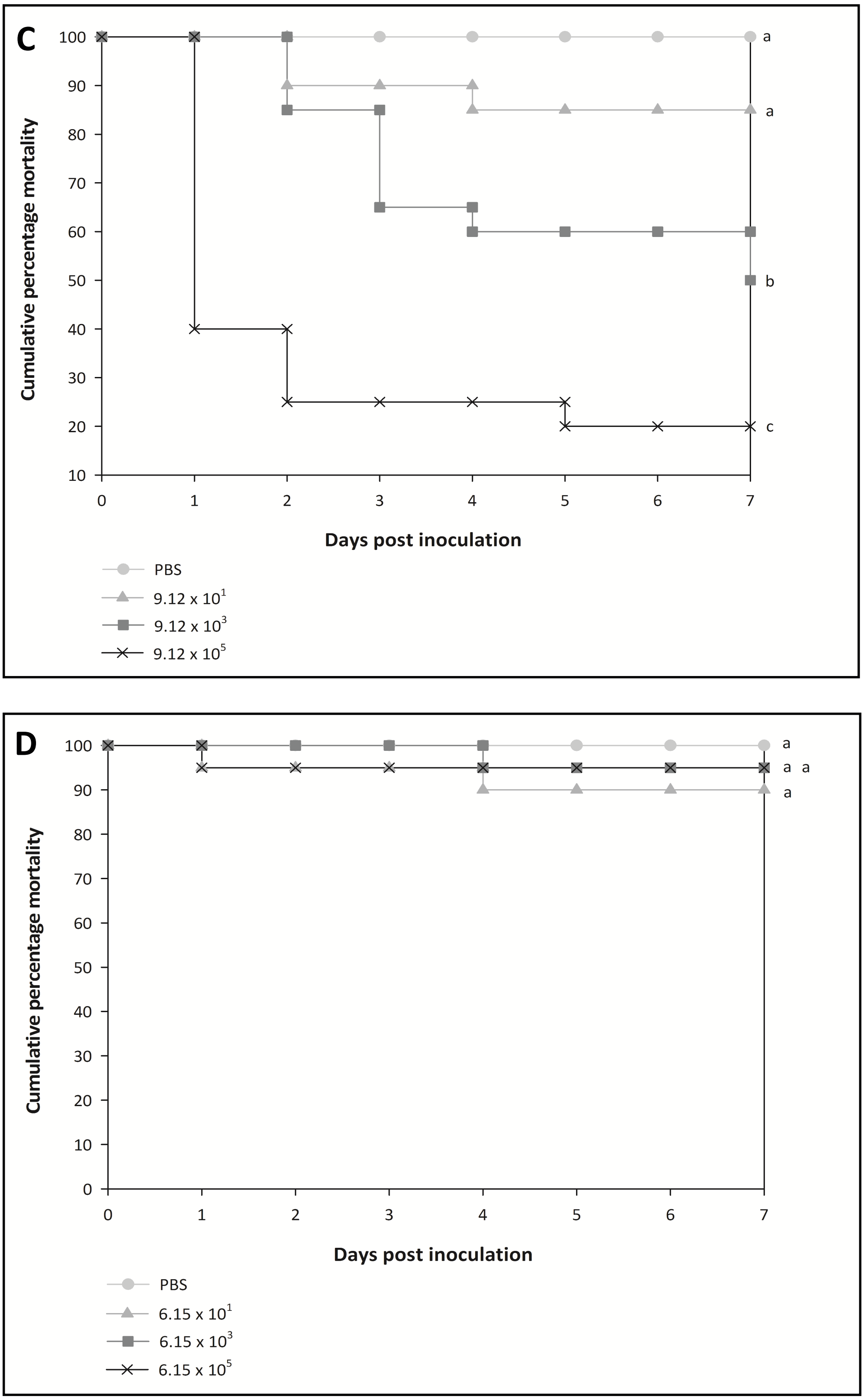

Figure 4.5 Kaplan-Meier survival curve for wax moth larvae inoculated with [C] *Streptococcus iniae* C and [D] *Streptococcus iniae* D at a range of concentrations over a 7 day period. Bacterial concentrations represent the concentration of bacterium per inoculum per larvae (cfu/ 10µl). Treatments that do not share a letter are significantly different. A value of $p < 0.05$ was considered significant and Holm's correction was applied to account for multiple comparisons.

4.3.2 Simultaneous challenge with of *Streptococcus agalactiae* and *Streptoccus iniae* in wax moth larvae

When the larvae were inoculated with a single bacterial species at various concentrations there was no significant difference in the overall percentage mortality between the three replicates (p = 0.571). There was a significant dose dependent difference within both *S. agalactiae* and *S. iniae* (p = 0.000) (Figure 4.6). There was no significant difference in mortality rates between the lower concentrations of *S. agalactiae* and *S. iniae* and between the middle concentrations of *S. agalactiae* and *S. iniae*. There was a significance difference in the mortality levels between the higher concentrations of *S. agalactiae* and *S. iniae* however, mortality levels were significantly higher than both the middle *S. agalactiae* and *S. iniae* concentrations (Figure 4.6).

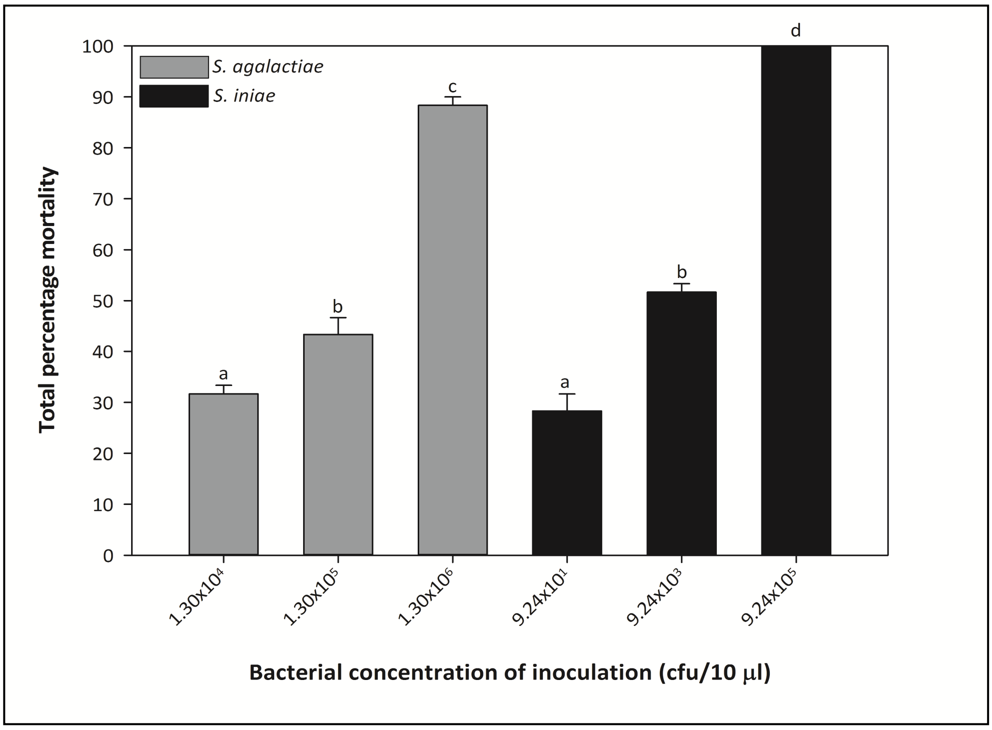

Figure 4.6 The total percentage mortality of wax moth larvae inoculated with a range of bacterial concentrations of *Streptococcus agalactiae* B and *Streptococcus iniae* C. Values represent mean percentage mortality ± S.E.M. Means that do not share a letter are significantly different ($p < 0.05$).

The results from the injection of the larvae with the combined bacterial species (co-infection) are presented in Table 4.3 and Figure 4.7. In some instances the overall percentage mortality did not differ when an additional pathogen was added to the inoculum. As shown in Table 4.3 a 30% mortality rate was observed when larvae were inoculated with [a] 1.3 x 10^4 cfu/inoculum *S. agalactiae* B [b] 9.24 x 10^1 cfu/inoculum *S. iniae* C and [c] a dual mixture of 1.3 x 10^4 cfu/ inoculum *S. agalactiae* B and 9.24 x 10^1 cfu/inoculum *S. iniae* C.

Table 4.3 Total percentage mortality of wax moth larvae injected with a mixture of PBS, *Streptococcus agalactiae* B and *Streptococus iniae* C in a range of concentrations (cfu/inoculum).

		***S. agalactiae* B**			
		PBS	**1.3 x 10^4**	**1.3 x 10^5**	**1.3 x 10^6**
***S. iniae* C**	**PBS**	0	30	45	90
	9.24 x 10^1	30	30	40	90
	9.24 x 10^3	50	25	40	75
	9.24 x 10^5	100	100	100	100

[NB] Shaded cells represent results of interest

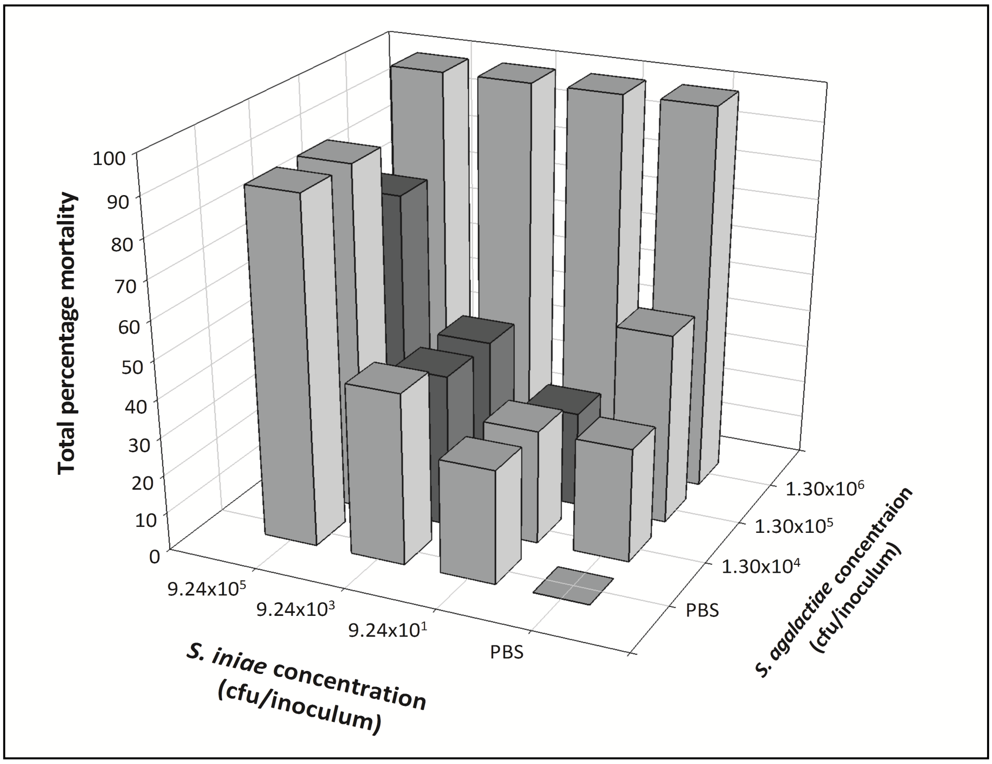

Figure 4.7 Total percentage mortality of wax moth larvae injected with a mixture of PBS, *Streptococcus agalactiae* B and *Streptococcus iniae* C in a range of concentrations (cfu/inoculum). [NB] Darker bars represent results of interest.

4.3.3 Competition assays determined by growth inhibition between *Streptococcus agalactiae* and *Streptococcus iniae*

In the competing drop colony assay the sequence of inoculation did not affect the outcome, as no inhibition of bacterial growth was observed with either early/late colonizers or when both species were inoculated at the same time. Similar to the competing drop colonies in the cross plate competition assay no inhibition of bacterial growth was observed in any of the test plates.

4.3.4 Assessment of *Streptococcus agalactiae* and *Streptococcus iniae* supernatant: Evaluation of bactericidal effect

(a) <u>Determination of viable counts of *S. agalactiae* and *S. iniae* after treatment with filtered supernatant.</u>

The average cfu (± S.D.) in the initial bacterial suspension was 28 ± 3.5 for *S. agalactiae* and 33 ± 13.4 for *S. iniae*. There was a > 500% increase in the cfu/ml of both *S. agalactiae* and *S. iniae* after incubation in TSB compared with the initial concentration of bacterial suspension. When both bacterial species were incubated in *S. agalactiae* supernatant neither bacterium showed significant growth. With the *S. iniae* supernatant *S. agalactiae* grew at a similar rate to the TSB control (Figure 4.8.A) indicating that *S. iniae* supernatant had no effect on the growth of *S. agalactiae.* Whereas cfu/ml dropped for *S. iniae* after incubation with *S. iniae* supernatant.

(b) <u>Filtered supernatant treated with proteinase K</u>

The average cfu (± S.D.) in the initial bacterial suspension was 19 ± 8.0 for *S. agalactiae* and 17 ± 5.5 for *S. iniae*. There was no significant growth of *S. iniae* and *S. agalactiae* in either the *S. agalactiae* supernatant or in the *S. agalactiae* supernatant treated with proteinase K.

When *S. iniae* was incubated with *S. iniae* supernatant treated with proteinase K there was a higher bacterial growth compared with the *S. iniae* supernatant alone (Figure 4.8.B). This suggested that treating *S. iniae* supernatant with proteinase K increased *S. iniae* growth. However, there was no significant growth of *S. agalactiae* in the *S. iniae* supernatant solution with or without proteinase K treatment. This indicated that treating *S. iniae* supernatant with proteinase K had little effect of the growth of *S. agalactiae.*

Although the values of the bacterial growth differed between experiments (a) and (b), as shown in Figures 4.8, the trends observed were consistent. There is one exception to this: the growth of *S. agalactiae* in *S. iniae* supernatant.

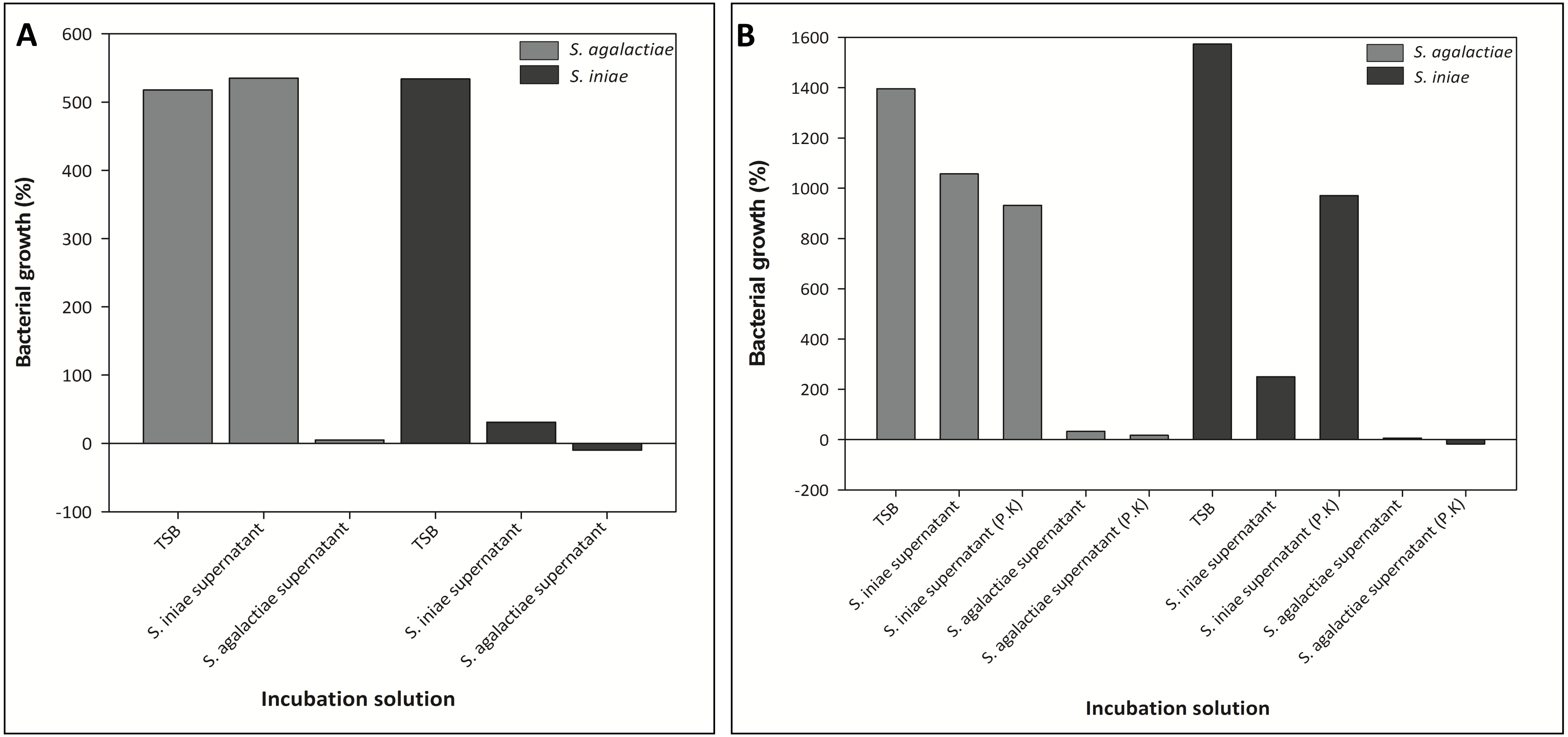

Figure 4.8 The average percentage growth of *Streptococcus agalactiae* and *Streptococcus iniae* after incubation in various bacterial supernatant solutions. Samples consisted of 50% *S. iniae* or *S. agalactiae* bacterial suspension and 50% incubation solution. [B] A repeat of [A] with additional incubation solutions. [P.K] denotes supernatants that had been treated with proteinase K.

(c) Filtered supernatant re-filtered with centrifugal filter units

For the first experiment the average cfu (± S.D.) in the initial bacterial suspension was 39 ± 2.1 and 67 ± 11.3 for *S. agalactiae* and *S. iniae* respectively. In the second experiment the average cfu (± S.D.) in the initial bacterial suspension was 43 ± 11 and 53 (± n/a) for *S. agalactiae* and *S. iniae* respectively. In both experiments when both bacterial species were incubated in *S. iniae* and *S. agalactiae* supernatants there was negligible growth. In the second assay, both *S. agalactiae* and *S. iniae* experienced bacterial death or negligible growth when incubated in *S. agalactiae* < 10 kDa supernatant and in *S. agalactiae* < 100 kDa supernatant. Both bacterial species also had a severely diminished growth rate in *S. iniae* < 10 kDa supernatant and in *S. iniae* < 100 kDa supernatant compared with the bacteria incubated in TSB.

As seen in Figure 4.9 varied results were obtained between the first and second assay when both *S. agalactiae* and *S. iniae* were incubated in bacterial supernatant fractions > 10 kDa and > 100 kDa. It was observed however, that generally in these incubation solutions the growth rate was higher than or similar to that seen when *S. agalactiae* and *S. iniae* were incubated in TSB.

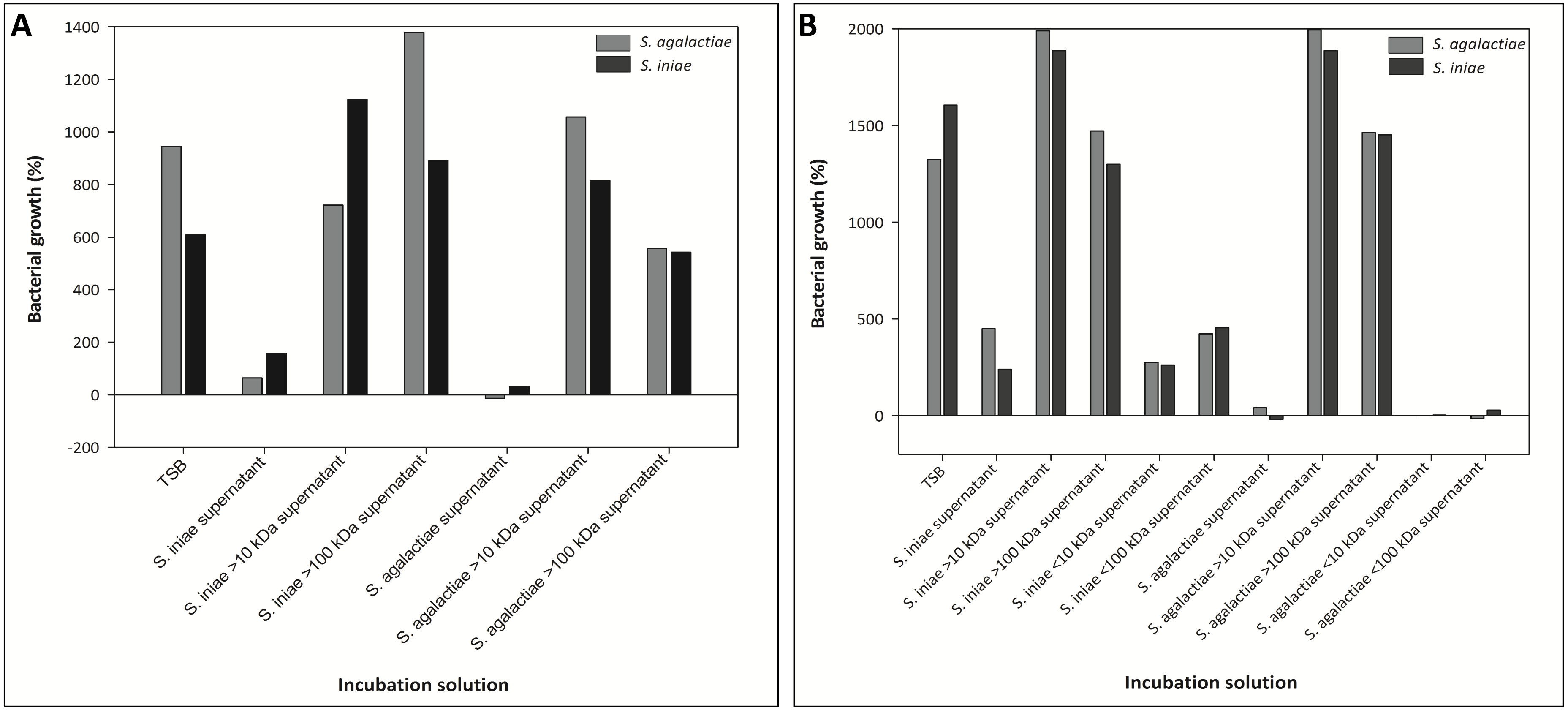

Figure 4.9 The average percentage growth of *Streptococcus agalactiae* and *Streptococcus iniae* after incubation in various bacterial supernatant solutions. Samples consisted of 50% *S. iniae* or *S. agalactiae* bacterial suspension and 50% incubation solution. [A] Bacterial supernatants were centrifuged using Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-10 membrane or Ultracel-100 membrane. The concentrated solutes produced (>10 and >100kDa supernatant solutions) were resuspended in TSB. [B] A repeat of [A] with the incorporation of the effluent supernatant that remained in the centrifuge tube after filtration. NB: <10 and <100 kDa supernatant solutions were not resuspended in TSB.

4.3.5 SDS-PAGE: Coomassie brilliant blue and silver staining

The SDS PAGE stained with coomassie brilliant blue showed a limited number of faint bands which were only visible in the concentrated solute samples (Figure 4.10.A). Analysis of the SDS PAGE with silver staining revealed a considerable higher quantity of bands ranging approximately from 10 – 100 kDa (Figure 4.10.B). Bands were not detectable or as visibly distinct in the < 10 kDa or < 100 kDa supernatant solutions compared with the concentrated solute samples. As seen in Figure 4.10.B there was some similarity in the profile of the protein bands between the two bacterial species but protein bands for *S. iniae* supernatant at ≈ 25 and 100 kDa were not seen in the *S. agalactiae* supernatant. Likewise, the two protein bands seen between ≈ 50 – 70 kDa in the *S. agalactiae* supernatant were not seen in the *S. iniae* supernatant.

Although different centrifugal filter units (10 kDa and 100 kDa) were used on the bacterial supernatants prior to SDS-PAGE both concentrated filtrates contained nearly identical profile of proteins.

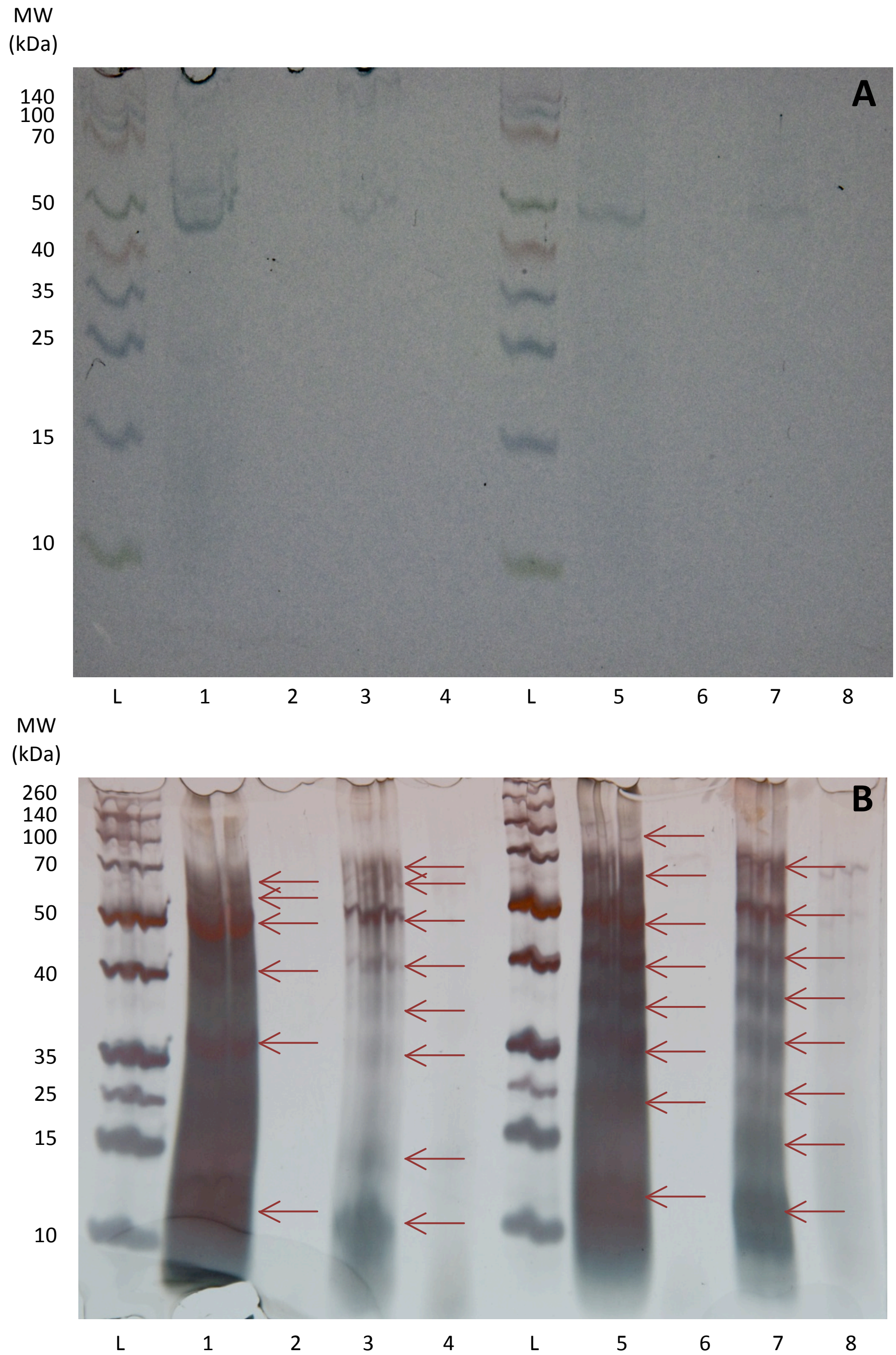

Figure 4.10 SDS-PAGE of *Streptococcus agalactiae* and *Streptococcus iniae* supernatant stained with [A] Coomassie blue and [B] silver stain. [L] Protein ladder [1] *S. agalactiae* > 10 K supernatant [2] *S. agalactiae* < 10 k supernatant [3] *S. agalactiae* > 100 k supernatant [4] *S. agalactiae* < 100 K supernatant [5] *S. iniae* > 10 K supernatant [6] *S. iniae* < 10 k supernatant [7] *S. iniae* > 100 k supernatant [8] *S. iniae* < 100 k supernatant. [MW] molecular weight. Arrows are used for lanes 1, 3, 5 and 7 to act as visual aids.

4.4 Discussion

Microbial co-infection studies are still in their infancy for studying aquatic disease outbreaks and yet they are highly likely to ocurr in the aquatic farming systems. Establishing a reproducible experimental animal challenge model is complicated and certainly there are few, if any, available for most of the aquatic diseases or farmed fish species. Understanding the complex interactions between micro-organisms has been achieved through quorum sensing. However the purpose of the study presented was to use *in vitro* screening methods and an alternative animal model to refine the subsequent *in vivo* fish challenges. It is difficult to predict the outcome of simultaneous pathogen exposure or how this fits within streptococcal disease dynamics. The alternative animal experimental model applied in this study was the wax moth larvae which have previously been used to test the virulence of several bacterial pathogens, producing results which correlate with infection models in mammals (Junqueira, 2012). This has been attributed to the similarities between the mammalian and insect innate immune response (Desbois and Coote, 2012; Kavanagh and Reeves, 2004). Insect immune defences rely on phagocytosis, clotting mechanisms, production of anti-microbial peptides and humoral factors (Desbois and Coote, 2012; Kavanagh and Reeves, 2004). All of which are similar with regard to the fish innate immune response (Segner *et al*., 2012; Zhu *et al*., 2013).

The application of alternative animal models for exploring aquatic pathogens is advancing (McMillan *et al*., 2013) but remains limited in application. The results from the study presented would suggest that for *S. agalactiae* the wax moth larvae were not a universally suitable replacement model for fish. Their use for *S. iniae* showed more promise as a potential screening tool; a clear difference in the virulence capabilities between the *S. iniae* strains was found using the larvae challenge model. Additionally, the wax moth larvae infection model was shown to be robust with good repeatability; there was little variation in mortality levels between replicates when injected with the same strain, again showing potential application for aquatic *S. iniae* research.

Whilst the direct replacement of *in vivo* fish studies with the wax moth larvae cannot be supported by the results presented in this study, their use as a pre-fish infectivity screening tool could contribute towards the refinement of fish experimental studies or a reduction in the number of strains tested in fish. Thus promoting the UK Home Office 3Rs in fish disease studies. Additionally, as suggested by Brennan *et al.* (2002), wax moth larvae may be used to test the virulence of mutants made through processes such as transposon mutagenesis to aid research into identification of virulence factors.

Standardised experimental conditions and protocols for the use of wax moth larvae during infection models have yet to be established making inter-laboratory comparisons difficult (Banville *et al*., 2012; Junqueira, 2012). Exposure to physical stress (Mowlds *et al.*, 2008) or mild thermal shock (Mowlds and Kavanagh, 2008) prior to inoculation and absence of nutrition (Banville *et al*., 2012) have all shown to alter the susceptibility of wax moth larvae to the pathogen. In this study, feed was not provided to the wax moth larvae. As per the protocol, the larvae were incubated at 4 °C prior to inoculation as this made the larvae less motile which eased inoculation proceedings. This enabled a more accurate administration of the bacterial inoculum whilst minimising the risk of damage to the larvae during the injection procedure. A uniform or standardised procedure nevertheless needs to be adopted regarding the use of wax moth larval allowing comparisons to be made between research studies and thus reducing the need to repeat investigations. Furthermore, studies need to provide more detailed descriptions of wax-moth larvae husbandry and specify whether nutrients are provided and in what form, as suggested by Banville *et al*. (2012), to facilitate comparisons between studies. This reasoning is applicable to most infection models. As a result of a large number of variables which are hard to control, challenge experiments can be difficult to standardise and replicate.

A novel approach utilising the wax moth larvae model was further explored when more than one pathogen was introduced to the host at the same time in a concurrent exposure. This was, again, approached in compliance with the 3Rs to reduce the number of fish studies performed and the larvae infectivity results indicated that a concurrent infection of *S. agalactiae* and *S. iniae* did not increase mortality rates. The larval mortality rate during a concurrent infection was equal to or lower than the mortality rates seen during separate individual bacterial challenges. This was an interesting observation as it suggested an impact on the pathogenicity of the two bacterial species when administered simultaneously.

It has been shown that bacteria have many active mechanisms which can impair or kill other microorganisms (Hibbing *et al.*, 2010). The more common products excreted include natural antibiotics, lytic agents, lysozymes and bacteriocins (Cascales *et al.*, 2007 cited in Riley, 2011). Such mechanisms are triggered when bacteria are in competition for the same pool of resources. In the cases where lower mortality levels were observed in the wax moth larvae when simultaneously infected with *S. agalactiae* and *S. iniae* it was hypothesised that *S. agalactiae* and *S. iniae* were in fact competing with one another, thus hindering each other's ability to cause mortality in the larvae. The hypothesis was developed that the pathogens may have been producing toxins such as bacteriocins. Riley (2011) defined bacteriocins as biological active protein moieties with a bacteriocidal mode of action. Researchers still debate whether these peptides only affect closely related microorganisms (Riley, 2011) or have a wider range of effect on unrelated microorganisms (Balciunas *et al.*, 2013). To test the competition/coexistence hypothesis between the two bacterial species investigated in this study, the cross-streak method and colony drop assays were performed under nutrient limiting conditions. It was found that there was no reduced growth of *S. agalactiae*, *S. iniae, L.*

garvieae and *E. faecalis* in any assay indicating that there was no competition between these pathogens within these test environments.

There are several possible explanations for such findings: [1] the half strength TSA may not have been a harsh enough environment to encourage adaptive strategies [2] different environments might induce different responses [3] that there is no interaction between any of those species tested or [4] the bacteria may participate in cooperative behaviour. Hypothesis [4] is supported by Kreth *et al*. (2005) who found that two other closely related species, *Streptococcus mutans* and *S. sanguinis* engaged in a multitude of antagonistic interactions temporally and spatially on dental biofilms. It was found that regardless of the bacterial species the early coloniser that occupied a particular niche inhibited the growth of the later coloniser (competition) whereas simultaneous colonisation by both bacterial species resulted in coexistence. Environmental factors were, however, shown to influence these interspecies interactions which included cell density, nutritional availability and pH of the growth medium. Consequently, the preliminary investigation in this study to examine *S. agalactiae* and *S. iniae* interactions may not have been appropriate to encourage noticeable signs of competition.

Both *S. agalactiae* and *S. iniae* were incubated with bacterial supernatants to further explore any bactericidal effects. The results showed that incubation in *S. agalactiae* supernatant considerably reduced the ability of both *S. iniae* and *S. agalactiae* to grow whereas *S. iniae* supernatant only had a negative effect on the growth of *S. iniae*. To try to understand this further three hypotheses have been developed, all of which may be interlinked:

(1) Signalling mechanisms

Control of gene expression in response to environmental conditions is a fundamental activity performed by many bacterial species. Sitkiewicz *et al*. (2009) performed global transcript analysis on *S. agalactiae* throughout mid-log to stationary growth phases *in vitro* and monitored the expression of genes and regulons. From this work it was shown that *S. agalactiae* can activate genes involved in the metabolism of nutrients and carbon sources, protect against changing pH and slow down cell division and decrease transcription and translation. Numerous other genes and regulons were shown to have been up or down regulated which included virulence factors and regulator/signal transduction systems (Sitkiewicz *et al*., 2009). An example of such is the *luxS* gene which has a function in quorum sensing; a bacterial cell-to-cell communication process involving the production and detection of extracellular signal molecules called autoinducers (Xavier and Bassler, 2003). Quorum sensing activity has shown to control behaviours such as antibiotic production, biofilm formation and virulence factor secretion (Rutherford and Bassler, 2012). Xavier and Bassler (2003) stated that some autoinducers can be used to respond to bacterial-densities, used for interspecies cell-to-cell communication and relay information about the fitness of the bacterial population. There is very little information regarding quorum sensing in *S. agalactiae* but the changes observed in transcript levels of the *luxS* gene observed in research by Sitkiewicz *et al*. (2009) indicates it may have some relevance for bacterial survival and/or fitness. Although the adaptive response of *S. agalactiae* was only studied between mid-log to stationary growth phases it is reasonable to assume that similar adaptive responses could occur at an early stage of bacterial growth. Consequently, in relation to this study, if gene expression and therefore signalling mechanisms were modified during the initial bacterial incubation in TSB this may affect the ability of the bacterium to initially grow when placed in the supernatant growth medium. The bacteria's gene expression may not be suitable for this new environment and a delay in growth could occur whilst the bacterial adjusts is gene expression.

(2) Production of extracellular products including bacteriocidal/bacteriostatic-like inhibitory compounds

As described previously, bacteria may produce extracellular products that have bacteriocidal/bacteriostatic properties. Furthermore, bacterial species such as *S. pneumoniae* and *Bacillus subtilis* have shown to partake in cannibalism and fratricide respectively (Be'er *et al*., 2009) which may be regulated by quorum sensing (Portugal, 2013). Autoinducers in Gram-positive bacterial quorum sensing are commonly oligopeptides (Portugal, 2013). During the initial growth of *S. agalactiae* and *S. iniae* in TSB any extracellular/autoinducers released for the purpose of regulating bacterial growth would have been produced in proportion to the bacterial density or to a threshold level. However, when the small number of bacterial cfu were subsequently incubated with the bacterial supernatant it is possible to assume the ratio of the extracellular products to bacterial density was now skewed. This could have severely hindered the bacteria's ability to grow. It is hypothesised that *S. agalactiae* produced an extracellular product with repercussions on intra and inter species growth whereas *S. iniae* produced an extracellular product which only restricted growth of its own species.

(3) Growth Kinetics

During growth, bacteria are continuously reacting and adapting to changing physical and chemical environmental conditions. Both *S. agalactiae* and *S. iniae* were grown to early-log phase in TSB before a bacterial suspension in saline was made which was subsequently mixed with a bacterial supernatant. Therefore the bacteria were, in principle, inoculated into a different culture medium and so may have required a period of adaptation to the new environment before accelerated growth could occur. This may have induced the bacterium into apparent lag or true lag phase of growth. Kun (2013) stated that true lag occurs when the culture is not able to grow at its maximum rate initially due to either (i) change in nutrient (ii) change in the culture conditions (iii) presence of an inhibitor or (iv) inoculum effect. This would subsequently result in negligible or no bacterial growth for period of time.

Both *S. agalactiae* and *S. iniae* were grown in TSB for an identical incubation time and based on previous growth curve studies this placed them in early-log phase. This ensured that the incubation between the two bacterial species was comparable and also that bacterial numbers in the culture medium at this time point was similar between the two species. This was in an attempt to ensure that nutrient content of the culture medium (and thus the bacterial supernatant) at this time point was similar between *S. agalactiae* and *S. iniae. Streptococcus agalactiae* grew in TSB and *S. iniae* supernatant at a similar rate, therefore the *S. iniae* supernatant evidently contained the necessary nutrient requirements required for bacterial growth and *S. agalactiae* was able to quickly adapt to a change in growth medium. However, as both *S. agalactiae* and *S. iniae* had reduced or no growth after incubation with *S. agalactiae* supernatant it may be possible that this was due to nutrient deficiencies in the medium. If the period of observation was extended, a resumption of growth would indicate a lag phase rather than a deficiency of nutrients.

To determine if the low bacterial growth was due to an extracellular protein a proteinase K treatment was incorporated into the experimental design. Proteinase K is commonly used to digest protein. It was found that the proteinase K treatment applied to the *S. iniae* supernatant resulted in higher *S. iniae* growth than in *S. iniae* supernatant with no treatment. Therefore, there may have been a protein produced by *S. iniae* with effects on conspecifics.

The reduced bacterial growth observed in *S. agalactiae* supernatant did not appear to be mediated by a protein product. However, some proteins have been found to be proteinase K resistant such as the bovicin HC5, a bacteriocin from *S. bovis* HC5 (Mantovani *et al*., 2002). Consequently, it cannot be conclusively stated that reduced growth observed in *S. agalactiae* supernatant was not due to a protein.

When centrifugal filter units were used on the bacterial supernatant the concentrated solutes that were produced were resuspended in TSB before being incubated with the bacteria. Generally, these incubation solutions produced higher bacterial growth than when the bacteria were incubated with TSB alone. When the bacteria were incubated in either [a] the supernatant that remained after the centrifugal filters units were used or [b] in the unfiltered bacterial supernatant, bacterial growth was significantly lower compared with the TSB control group. There was obviously a nutritional/compositional difference between all these aforementioned incubation solutions for the main reason that fresh TSB was used in the preparation of some of these solutions. This makes analysis of the results difficult. In regards to *S. agalactiae* supernatant the incorporation of TSB into the incubation solution appears to either replenish a limited nutrient element within the incubation solution that was restricting bacterial growth or it diluted out the extracellular compound that was inhibiting bacterial growth.

Extracellular products (ECP) are produced by a diverse range of pathogenic microbes and play a critical role in the pathogenesis of infection (Lei *et al*., 2000; Madureira *et al*., 2007). Identification and characterisation of ECP in other *Streptococcus* species, such as Group A *Streptococcus* (GAS), has been well researched and three general categories of extracellular proteins have been established. Extracellular proteins from GAS include streptococcal pyrogenic exotoxins, the virulence factors M protein and C5a peptidase (Lei *et al*., 2000). Research on *S. agalactiae* and *S. iniae* ECP is somewhat lacking in comparison. However, antigenic and virulence proteins have been identified as ECP in both *S. agalactiae* and *S. iniae* (Eyngor *et al*., 2008; Eyngor *et al.*, 2010; Madureira *et al*., 2007; Nho *et al*., 2011). Klesius *et al*. (2007) also identified ECP and proposed that they were likely to be involved in the proinflammatory responses of macrophages to *S. agalactiae* and *S. iniae* infections. The molecular weight of a few ECP has been determined and include 7.54, 45, 47, 54, 55, 75 kDa

(Klesius *et al.*, 2007; Madureira *et al.*, 2007; Pasnik *et al.*, 2005) for *S. agalactiae* ECP and 19.2 kDa (Klesius *et al.*, 2007) for *S. iniae* ECP.

In this study, protein bands within the bacterial supernatant were only visibly distinct on a SDS-PAGE when centrifugal filter units were used to concentrate the supernatant proteins and silver staining was performed on the gel. This was not surprising as silver staining provides excellent sensitivity and is 30 – 100 times more sensitive than colloidal Coomassie Blue (Chevallet *et al*., 2006). Comparison between the protein characterisation from *S. agalactiae* and *S. iniae* supernatant showed that there was a level of similarity between them. This was to be anticipated as *S. agalactiae* and *S. iniae* are closely related microorganisms. However, there were differences in the number of protein bands visible for each bacterial species and their molecular weight. This may explain why the contents of *S. agalactiae* supernatant have intra and inter species effects whereas *S. iniae* supernatant is intra species specific; if in fact the reduced growth seen was mediated by a protein.

The mechanisms that regulate bacterial growth, including the production of extracellular products and quorum sensing, are complex and not well understood for aquatic pathogens. Signals of growth regulation and interspecies communication has been discussed previously (Hayes and Low, 2009; Ryan and Dow, 2008), however, research into *S. agalactiae* and *S. iniae* growth and communication is scarce. The identification and characterisation of the ECP found in *S. agalactiae* and *S. iniae* supernatant was beyond the scope of this study. Thus no definitive explanations can be made for the reduced bacterial growth observed when *S. agalactiae* and *S. iniae* were incubated in *S. agalactiae* supernatant. However, *S. iniae* does appear to produce a protein that effects intra-species growth. Further studies are necessary to understand the functional role of any ECPs and establish their importance in regulating growth, cell communication and in pathogenicity.

Competition between *S. agalactiae* and *S. iniae in vitro* was inconsistent in different experimental systems. Results indicate that there was either no interaction between bacterial species or they coexisted in competition assays. In the *in vitro* model utilising the wax moth larvae it was established that these model organisms have potential value in aquatic pathogenesis research. However, every application requires thorough validation and further research incorporating histopathology and gene expression would be required to make definitive confirmation. During a simultaneous infection with *S. agalactiae* and *S. iniae* total levels of larvae mortality were lower than expected which may have resulted from the pathogens interacting with one another in a competitive manner. As highlighted by Evans and Rozen (2012) key components to understanding bacterial diseases are the bacteria-bacteria interactions and bacterial-host interactions. Consequently, further studies of simultaneous infection with these two organisms were carried out *in vivo* using Nile tilapia.

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Chapter 5

Streptococcus agalactiae and Streptococcus iniae infection models in Nile tilapia

Some of work presented in this chapter has been published in *Aquaculture Research*. Featherstone, Z.L., Turnbull, J.F., Auchinachie, N.A. and Crumlish, M. (2015) Evaluation of visible implant elastomer (VIE) tags for pathogenesis research in Nile tilapia (*Oreochromis niloticus*). *Aquaculture Research* DOI: 10.1111/are.12688.

5.1 Introduction

The role of the individual fish in disease dynamics studies are complicated in aquatic systems. Tagging systems are applied in aquatic studies to assist in the rapid identification of individuals within a group. A visible implant elastomer (VIE) tag is a two-part mixture that once injected cures into a pliable, biocompatible solid mark (NMT, 2008) and has been applied in fish research to identify or mark individual animals. Once implanted beneath transparent or translucent tissue the VIE tag is visible to the naked eye under ambient light however tag visibility is enhanced through the use of UV illumination (FitzGerald *et al*., 2004). Whilst various other tagging methods have been used in aquaculture studies (e.g. fin clipping, spine punching and the attachment of tags) the injection of subcutaneous VIE tags has been tried on a broad range of finfish species as well on crustaceans, reptiles and amphibians (NMT, 2008). VIE tagging has been successfully applied for marking species such as Atlantic salmon (*Salmo salar*) (FitzGerald *et al*., 2004), brook trout (*Salvelinus fontinalis*) (Josephson *et al*., 2008) and several species of coral reef fish (Frederick, 1997). The benefits of VIE tags include rapid application, good externally visibility, high retention rates over time whilst having negligible effect on fish survival, growth and behaviour (NWT, 2008). However, the success of VIE tagging depends on the fish species (Reeves and Buckmeier, 2009), tagging location (Hohn and Petrie-Hanson, 2013), fish size (Close and Jones, 2002), study duration (Wagner *et al*., 2013), tagging experience of the operator (Hohn and Petrie-Hanson, 2013) and colour of the tag (Brennan *et al*., 2007; Curtis, 2006).

The VIE tagging system has a wide application for use in aquatic animal studies yet little information can be found regarding the use of these tags in pathogenesis research. Fish have been previously tagged for co-habitation challenges and vaccination trials (Alcorn *et al*., 2005; Klesius *et al*., 2006; Lin *et al*., 2006), however, its application in bacterial challenge experiments is lacking. Pathogen challenge studies are often performed under experimental conditions with groups of fish, susceptible to the microbial pathogen. However, disease establishment is a complex process as individuals in the group will become infected at different times. Consequently clinical signs of disease which may be presented could be missed unless the fish are under constant observation. This can prove to be impractical, labour intensive, and alter the behaviour of the fish as they can respond to human presence. If fish could be individually marked and monitored using time-lapse photograph or video recording the behaviour of fish could be assessed without being intrusive. Being able to track individuals within a group would provide valuable information on disease progression and improve our understanding of microbial pathogenesis in fish.

The aim of this study was to perform a series of bacterial experimental challenges and assess the efficacy of the VIE tagging system in identification of the individual animals during these *in vivo* experiments. Nile tilapia (*Oreochromis niloticus*) would receive either *Streptococcus agalactiae*, *S. iniae* or a combination of both organisms. The outcomes of this study would be used to formulate an experimental design examining sequential challenge models in tilapia.

5.2 Materials and methods

5.2.1 Streptococcal infection models

5.2.1.1 Fish

All of the fish used in these experiments were Nile tilapia (*O. niloticus*) provided from in-house stocks at the Institute of Aquaculture, University of Stirling. The fish groups were from different populations, whereby fish had different parental lines, and where of mixed sex. The animal and husbandry parameters are summarised in Table 5.1.

All fish were maintained in 10 l plastic tanks with continuous flow-through water at maximum flow of 0.7 l/minute and an air stone for aeration. Fish were starved for 24 hours prior to bacterial exposure then subsequently fed with a commercial diet from Skretting (Nutra Plus) to apparent satiety twice daily. The light regime used was a 12 hour light: 12 hour dark cycle.

Table 5.1 Details of the fish groups used for bacterial challenge models

	S. iniae				***S. agalactiae***			***S. iniae* and *S. agalactiae***
Approximate age (months)	4				7			8
Number of tanks used	4				3			1
Water temperature (°C)	28 ± 0.0				25.6 ± 0.5			28.3 ± 3.0
Number of fish per tank	20				20	20	10	10
Weight (g)	22.0 ± 2.1	22.5 ± 2.1	22.1 ± 3.1	*22.8 ± 2.1	30.3 ± 4.7	30.2 ± 4.8	*40.4 ± 2.8	28.3 ± 3.0

Numbers represent average value ± standard deviations when applicable [*] control tanks that received no bacterial challenge.

5.2.1.2 Preparation of bacterial inoculum

Passaged isolates of *S. agalactiae* B and *S. iniae* C (Table 3.1) were sourced from the culture collection at Institute of Aquaculture, University of Stirling and revived on tryptone soya agar (TSA) (Oxoid Ltd, Basingstoke, UK) as previously described (Section 2.2.1). A bacterial suspension was made in 0.85% saline using the method described in Section 3.2.7. Serial dilutions were then performed using sterile physiological saline as the diluent and provided expected bacterial concentration of:

[1] *S. iniae*: 1 x 10^6 cfu/100 µl, 1 x 10^7 cfu/100 µl and 1 x 10^8 cfu/100 µl

[2] *S. agalactiae*: 2 x 10^7 cfu/100 µl and 2 x 10^8 cfu/100 µl

[3] *S. agalactiae* and *S. iniae* combined: *S. iniae* 5 x 10^6 cfu/50 µl and *S. agalactiae* 1 x 10^7 cfu/50 µl

A viable cell count was carried out to confirm the actual bacterial concentration as described by Miles and Misra (1938) and in Section 3.2.4.

5.2.1.3 Bacterial challenge in tilapia

The bacterial challenges for *S. agalactiae, S. iniae* and *S. agalactiae/S. iniae* combined were performed independently from one another and all treatment groups were kept in separate tanks. For inoculation, fish were removed from their tanks and lightly anaesthetised with 50 ppm benzocaine solution (Sigma-Aldrich, Buchs, Switzerland). Each fish was injected intraperitoneally (i.p.) with 100 µl of the *S. agalactiae, S. iniae* or *S. agalactiae/S. iniae* combined suspension. Fish were allowed to recover from anaesthesia before being returned to the tanks and subsequently monitored for 10 days. If any fish showed signs of morbidity during this time they were immediately euthanized with an overdose of 10% benzocaine solution. Control groups for the *S. agalactiae* and *S. iniae* challenge received no bacterial challenge but did receive an i.p. injection of sterile 0.85% saline (100 µl/ fish).

5.2.1.4 Bacterial recovery and identification

Bacteria were aseptically recovered from moribund or dead fish. This was achieved by inserting a sterile plastic loop into the kidney of the fish and inoculating a TSA plate which was then incubated at 28 °C for 48 hours. Colony growth was purified as required and subsequently identified using a Gram stain, oxidase (Sigma-Aldrich, Buchs, Switzerland) and motility test as described by Frerichs and Millar (1993). A Slidex Strepto-kit (biomérieux, Marcy l'Etoile, France) was also performed according to the manufacturer's instructions. DNA extraction and PCRs were performed as described previously (Sections 2.2.5 and 2.2.6) using the primers F1/IMOD and LOX-1/LOX-2, which detects *S. agalactiae* and *S. iniae* respectively.

For the *S. agalactiae/S. iniae* combined challenge, a small sample (≈ 0.03 g) of kidney was taken aseptically from 4 fish and stored at -70 °C until required. DNA extraction was performed on the tissue samples using RealPure DNA extraction kit (Thistle Scientific) according to the manufacturer's instructions and then a duplex PCR was performed as described (Section 2.2.6.2) using illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Buckinghamshire, UK).

5.2.1.5 Clinical signs and histopathology

In this study clincal signs of disease denotes behavioural abnormalities, changes in external appearance and any post mortem findings such as changes to internal organs and histopathology findings. Dead or moribund fish were examined for any external or internal gross clinical signs of disease. The brain, eyes, gills, heart, kidney, liver and spleen were also removed from these fish and fixed in 10% (v/v) neutral buffered formalin for histopathology. The tissues were processed using standard protocols (Shandon Citadel 2000 tissue processing machine, Thermo Scientific, Hempstead, UK), embedded in paraffin wax blocks (Leica Histoembedder, Leica Microsystems Ltd, Milton Keynes, UK), and 3 µm sections cut (Leica RM 2035 microtome, Leica Microsystems Ltd, Milton Keynes, UK). The tissue sections were then stained using Gram staining (See appendix), for the detection of bacteria, and haematoxylin and eosin (H&E) staining (See appendix) for histopathology assessment. Images were captured using a Zeiss AxioCam MRc digital camera on an Olympus BX51 microscope. On images taken, arrows were added to indicate the presence and location of bacteria. Prior to the *S. iniae* challenge two healthy tilapia were sampled to provide comparative histopathology samples.

5.2.1.6 Immunohistochemistry

Immunohistochemistry was used as a means of observing and determining the location of bacteria in the organs of presumptively infected fish. Immunohistochemical assays were performed on deparaffinised, rehydrated 3 µm sections of tissue samples from the *S. iniae* and *S. agalactiae* challenge.

For the *S. iniae* challenge the anti-*Streptococcus iniae* monoclonal antibody (Aquatic Diagnostic Ltd, Stirling, UK) was used according to manufacturer's instructions. Tissue samples obtained from healthy fish were used as a negative control. Positive control samples consisted of tissue sections from a diagnostic clinical case from a natural outbreak of *S. iniae* infection. These were incubated with the reconstituted monoclonal antibody and PBS separately. *Streptococcus iniae* antigens were visualised as golden brown in colour against a bluish background.

For the *S. agalactiae* challenge immunohistochemistry was performed using primary rabbit anti-*S. agalactiae* polyclonal antibody (Abcam, Cambridge, UK) on samples from the *S. agalactiae* challenge according to the protocol described by Delannoy (2013). Negative controls were prepared by substituting the primary antibody with normal rabbit serum diluted 1:200. Tissue samples obtained from healthy fish were used also used as a negative control. To test the specificity of the antibody samples from the *S. iniae* challenge were included in addition to samples from a *S. iniae* diagnostic case from the Institute of Aquaculture, University of Stirling. *Streptococcus agalactiae* antigens were visualised as red in colour against a purple background.

5.2.2 VIE tag administration in Nile tilapia

5.2.2.1 Tagging protocol

VIE tags were purchased from Northwest Marine Technology, Inc. (Washington, USA) and were prepared according to the manufacturer's instructions. Two tag colours were tested (red and green) and were administered at five different locations in each fish, on the nasal, branchiostegal rays inside left operculum, base of the pectoral fin, upper abdomen and lower abdomen (Figure 5.1). Pilot studies had shown that these were the most suitable locations for tag administration as other locations including the upper jaw, dorsal fin, caudal fin, base of causal fin and anal fin were found to be unsuitable. This was due to either the physical difficulty in tagging in that location or the VIE tag was not retainable under the skin.

Fish were anesthetised in a 10% benzocaine solution and elastomer implanted with an insulin syringe mounted with a 29 gauge needle (BD, Oxford, UK). For each tagging site the needle was inserted below the skin and any excess elastomer removed before fish were gently placed into a recovery tank.

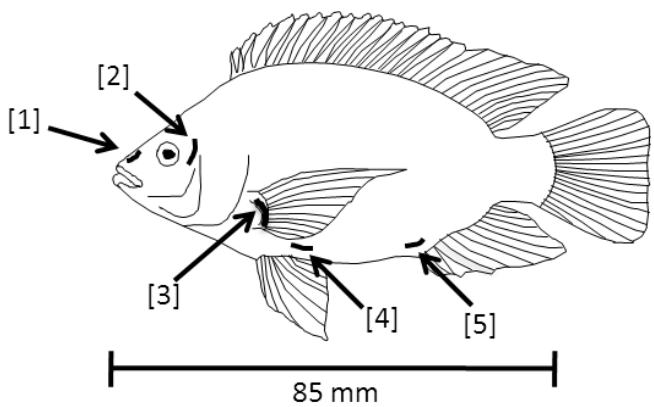

Figure 5.1 Locations of VIE tags on Nile tilapia. Tags were placed below the skin on the [1] nasal [2] branchiostegal rays inside the operculum [3] base of the pectoral fin [4] upper abdomen and [5] lower abdomen.

5.2.2.2 Fish

Tilapia were from a single population, of mixed sex at 26 ± 3 g in weight and approximately 4 months in age. All fish were maintained as previously described (Section 5.2.1.1) with the addition that the amount of feed used during each meal was measured. The water temperature was maintained at 24 ± 0.5 °C for the duration of the 21 day study period.

5.2.2.3 Study design

Tank A and tank B consisted of VIE-tagged fish where 5 fish received red VIE and 5 fish received green VIE as described above. In tank C were the non-tagged control fish which were handled in the same manner, with a clean needle placed under the skin in the same locations as fish in tanks A and B but no elastomer was implanted. Each group had a total of 10 fish. During the study, fish were removed from the holding tank, lightly anesthetized as previously described and measurements taken of their wet weight, length and tag retention. Such measurements were taken on day 0, 7, 14 and 21 post-VIE tagging. Fish length was measured from the tip of the snout to the base of the caudal fin (Figure 5.1). Tag visibility was assessed based on methods described by Leblanc and Noakes (2012) and Zakeś *et al*. (2013). Tags were assessed under natural light, by the same observer from an approximate distance of 30 cm, whilst the fish was out of water. If any portion of the tag was detectable the tag was measured as visible. On day 21 tag visibility was also assessed under UV light (blue light with amber glasses). All measurements were recorded and analysed using the statistical software JMP, through General Linear Models.

5.2.3 Application of VIE tags during bacterial challenge

5.2.3.1 Fish

The fish were provided as described above from the same population except that they were 30 ± 5 g in weight, approximately 5 months of age. The fish husbandry conditions were the same as previously described (Section 5.2.1.1) with the exception that fish were kept on a 24 hour light regime so fish could be easily monitored throughout the experimental challenge.

5.2.3.2 Tagging 7 days prior to bacterial inoculation

Passaged isolates of *S. agalactiae* B and *S. iniae* C were prepared as previously described (Section 5.2.1.2). The bacterial concentrations used were *S. iniae* 1 x 10^7 cfu/100 µl and *S. agalactiae* 2 x 10^7 cfu/100 µl.

The treatment groups used in this study [A1-5] are summarised in Table 5.2. A total of 50 fish were used with 5 treatment groups and 10 fish per group, all kept in separate tanks. Two treatments groups were tagged with VIE then 7 days later inoculated with either *S. agalactiae* or *S. iniae*. Two treatment groups were also inoculated with *S. agalactiae* or *S. iniae* but received no tagging prior to this. The remaining group acted as a control and were not tagged nor exposed to bacteria. Tagged fish received either a red or green VIE tag in one of the five locations shown in Figure 5.1 thus providing 10 individual tags. Tags were placed on both sides of the body so fish could be constantly monitored and identified. For the bacterial challenge each fish was i.p. injected with 100 µl of the *S. agalactiae* or *S. iniae* suspension. The control group each fish receive an i.p. injection 0.85% saline (100 µl/fish).

Fish were observed 2 – 4 times daily for 10 days. For uninterrupted monitoring of the fish a time-lapse photography series was set up. This consisted of a Nikon D300s camera with a 60 mm micro lens taking a photograph every 60 seconds using the software Triggertrap intervalometer/timer on an Apple iPad mini. The time lapse photographs were then compiled to form a video with sequence software on an Apple imac. Any moribund or dead fish were examined for internal and external clinical signs of disease. Bacteria were recovered as described previously and identified by Gram stain, oxidase, motility and Slidex Strepto-kit.

5.2.3.3 Tagging at same time as bacterial inoculation

The treatment groups used in this study [B6-10] are summarised in Table 5.2. This study was a repeat of the aforementioned study (Section 5.2.3.2) with a few exceptions. In this study fish were tagged at the same time as the bacterial inoculation was administered rather than 7 days prior. In addition, the bacterial inoculums used in this study were prepared from bacteria successfully recovered and identified from treatment groups A1-5. A time-lapse photography series was set up using GoPro Hero 2 cameras with a GoPro app on an iPad mini that took an image at 20 second intervals. A video was formed as described above.

Table 5.2 Description of the treatment groups used to investigate the application of VIE tags during bacterial challenges

Treatment group		Pathogen administered	VIE tagging
A	1	*S. agalactiae*	✓ 7 days prior to bacterial inoculation
	2	*S. iniae*	✓ 7 days prior to bacterial inoculation
	3	*S. agalactiae*	×
	4	*S. iniae*	×
	5	Saline control	×
B	1	*S. agalactiae*	✓ at same time as bacterial inoculation
	2	*S. iniae*	✓ at same time as bacterial inoculation
	3	*S. agalactiae*	×
	4	*S. iniae*	×
	5	Saline control	×

5.3 Results

5.3.1 *Streptococcus iniae* challenge model

The rate of morbidty and mortality in the exposed fish group occurred in a concentration-dependent manner whereby a higher concentration of bacteria resulted in a higher percentage of moribund or dead fish (Figure 5.2). Bacteria were successfully recovered and identified as *S. iniae* from all moribund or dead fish. There were no mortalities in the control group. No bacteria was recovered from the surviving fish inoculated with 6.12 – 6.97 x 10^7 cfu/100 µl of *S. iniae* sampled at day 10 or from control fish.

Examples of gross external and internal clinical signs of disease exhibited during the challenge model are shown in Figure 5.3. Clinical signs observed included lethargy, erratic swimming, opaque eyes, curvature of the spine and haemorrhaging around the base of fins; internally splenomegaly was observed grossly. Many of the fish sampled showed post-mortem decay and were not suitable for histopathology investigations. From the samples that could be used, *S. iniae* presented with necrosis and thrombosis in some tissues such as the spleen, liver and gills (Figure 5.4). There were relatively few bacteria found within organs examined (Figure 5.4). This was particularly noticeable when compared with samples from the *S. agalactiae* challenge (Figure 5.8).

The presence and location of the bacteria was confirmed with tissue Gram stains and immunohistochemistry. When immunohistochemistry was performed, the bacteria appeared as golden brown, which indicated a positive result for *S. iniae.* The staining would suggest more extensive deposits of *S. iniae* antigens outwith the location of intact bacterial cells. However, there were also pockets of bacteria that were not stained (Figure 5.5). In the positive control clinical case there was also a positive staining for *S. iniae*; membrane bound melanin granules were also stained but were visually distinguishable from bacterial cells as they are significantly larger in size (Figure 5.5). Negative controls from healthy fish or infected fish with PBS substitute showed no golden brown stained antigens or background staining.

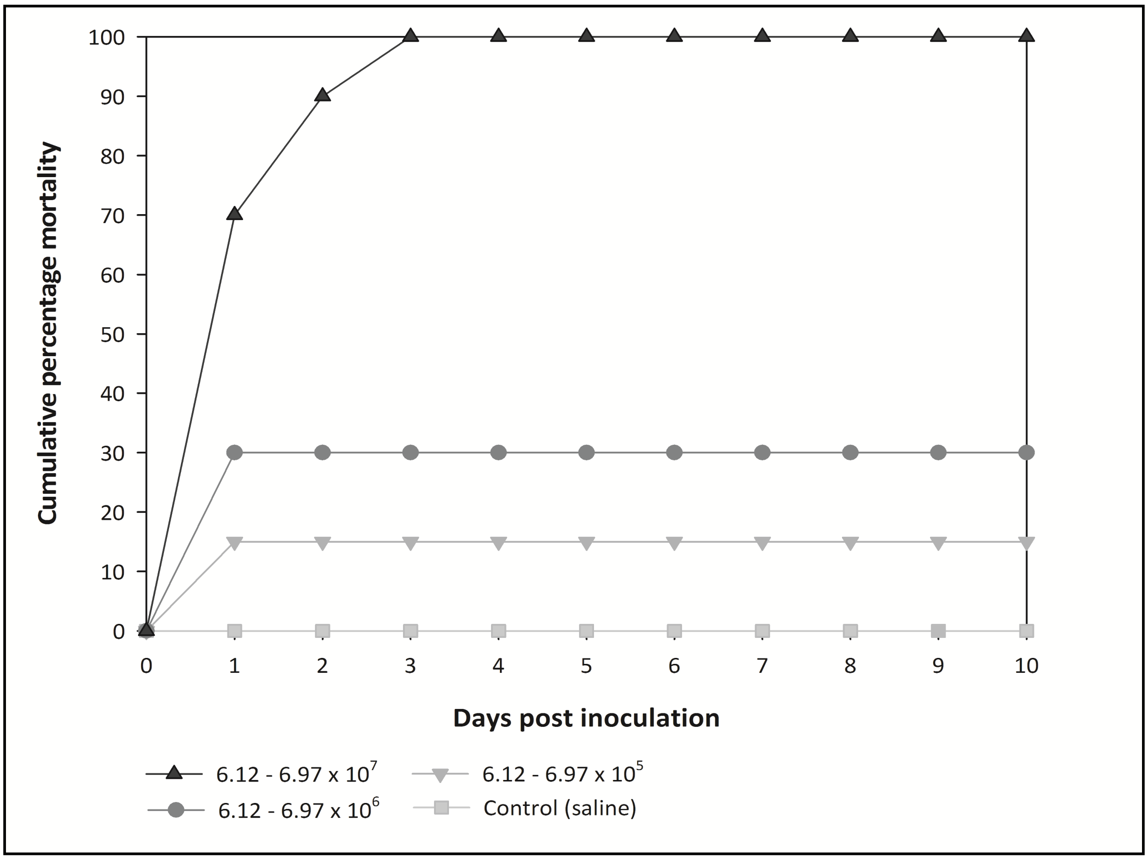

Figure 5.2 The cumulative percentage mortality of tilapia injected intraperitoneally with different *Streptococcus iniae* concentrations. Inoculation concentrations are represented as the number of colony forming units of bacteria per inoculum per fish. The bacterial concentration is presented as a range based on the viable cell count results.

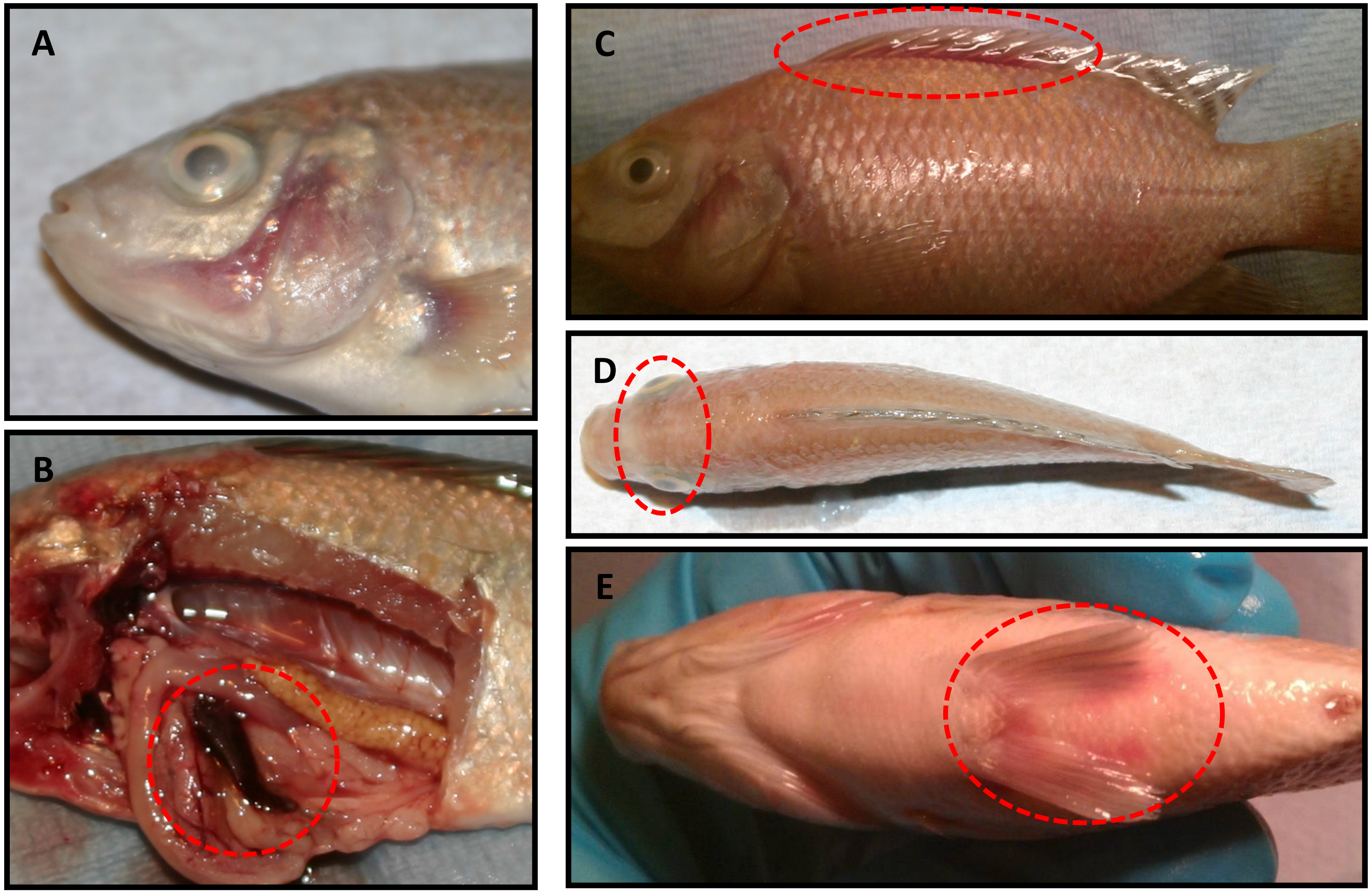

Figure 5.3 Dead or moribund fish inoculated with *Streptococcus iniae* 6.12 – 6.97 x 10^7 cfu/100 µl showing gross external and internal clinical signs of disease. [A] Opaque eye [B] Enlarged spleen [C] Haemorrhaging at the base of the dorsal [D] Curvature of the spine and unilateral opacity of the eyes [E] Haemorrhaging around the pelvic fins and on abdomen.

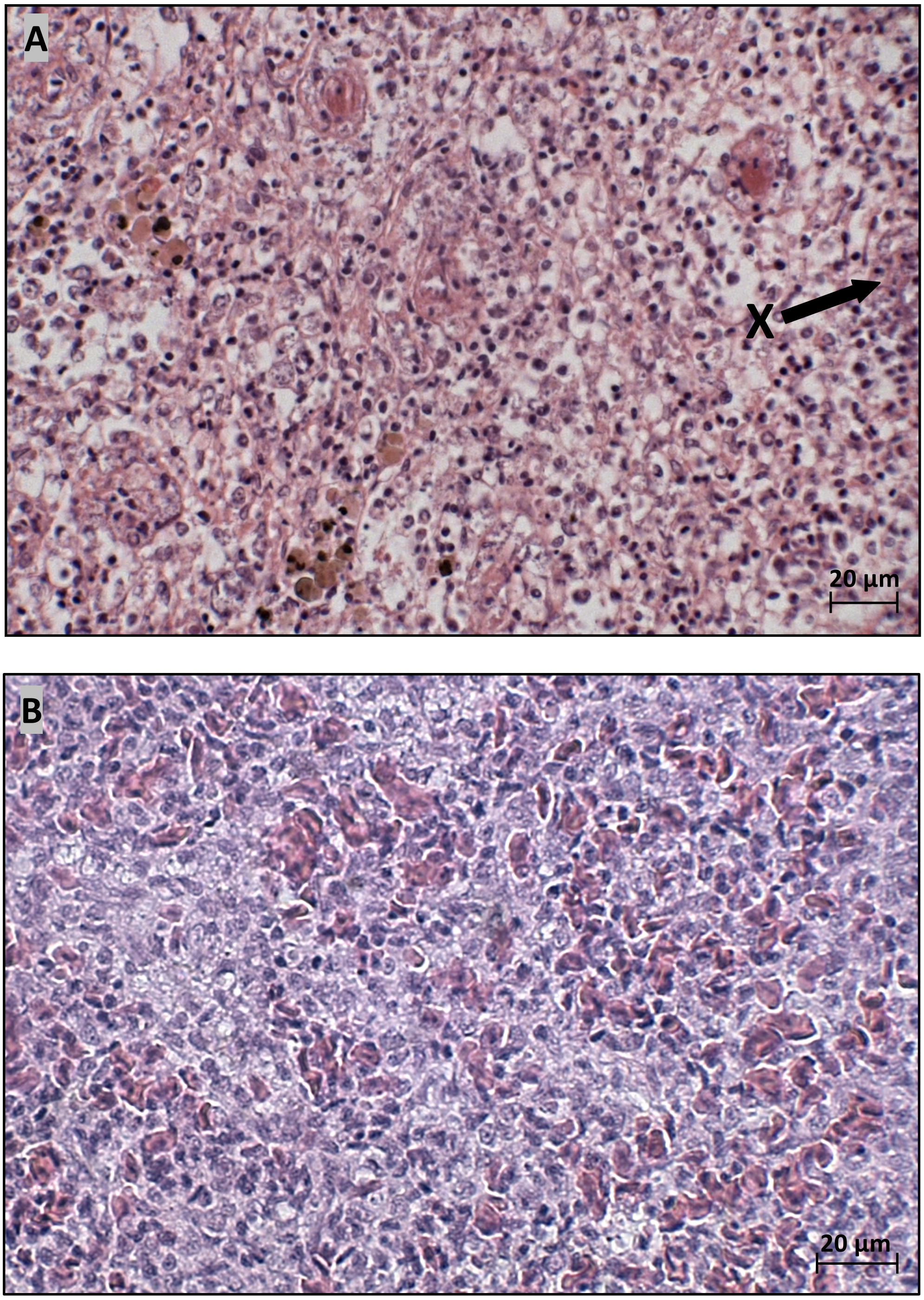

Figure 5.4 Histopathological changes in tilapia experimentally infected with *Streptococcus iniae* (H&E). [A] The spleen showing signs of extensive acute necrosis but compared with *Streptococcus agalactiae* relatively few bacteria and some cellular inflammatory response (X). [B] The spleen from a health tilapia sampled prior to the *S. iniae* challenge.

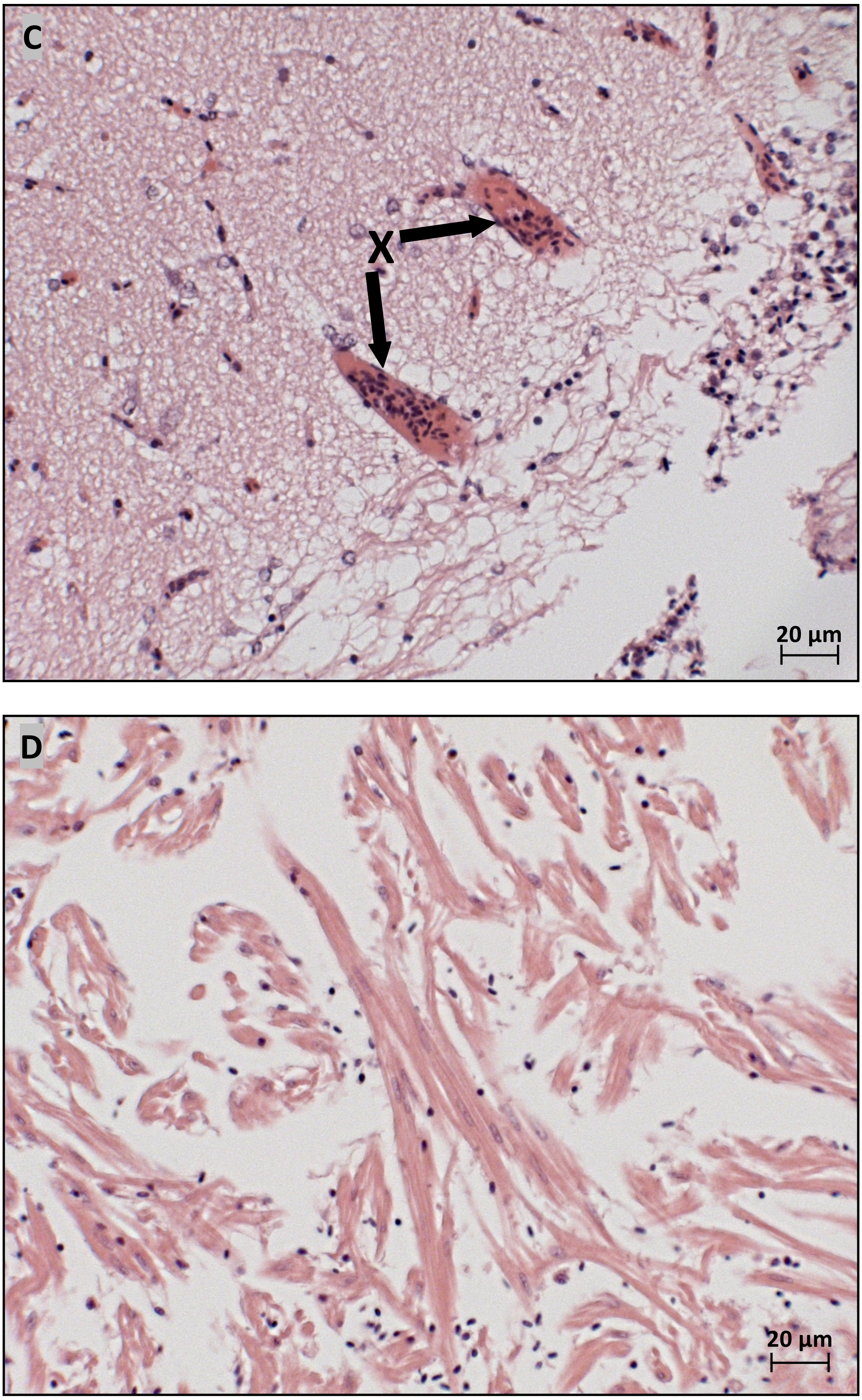

Figure 5.4 (continued) Histopathological changes in tilapia experimentally infected with *Streptococcus iniae* (H&E). [C] The brain with no evidence of bacteria but organising thromboses in the blood vessels (X). [D] The heart with no evidence of bacteria.

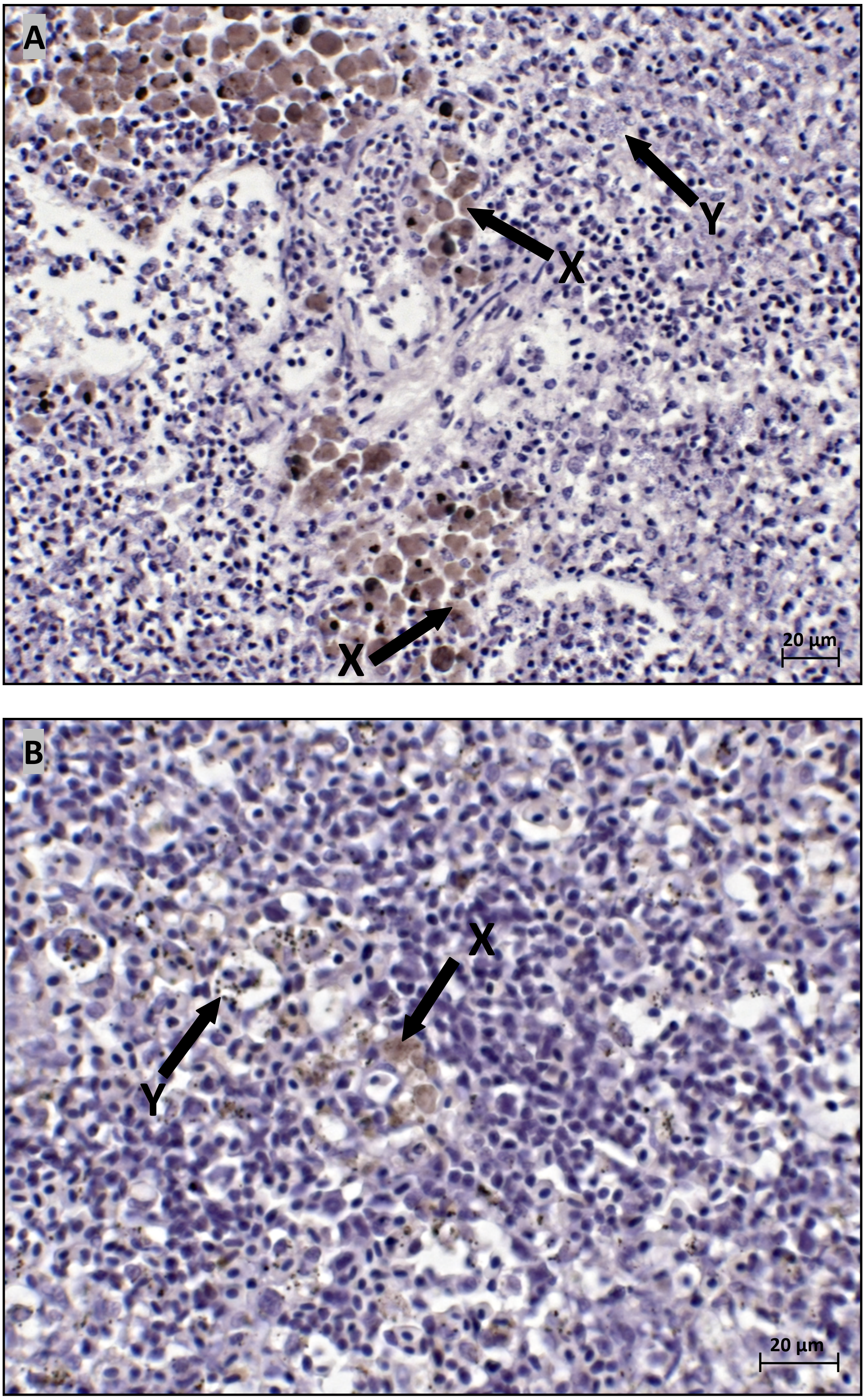

Figure 5.5 Immunohistochemistry in the spleen of tilapia infected with *Streptococcus iniae* using the anti-*Streptococcus iniae* monoclonal antibody. [A] Tilapia from this study experimentally infected with *S. iniae*. There are areas of postive staining assocaited with bacterial antigens (X); this staining would suggest more extensive deposits of *S. iniae* antigens outwith the location of intact bacterial cells. However, there are also pockets of bacteria with no staining (Y). [B] Fish (species unknown) from a clinical case naturally infected with *S. iniae.* Again there are areas of postive staining assocaited with bacterial antigens (X) but membrane bound melanin granules have also been stained (Y).

5.3.2 *Streptococcus agalactiae* challenge model

The cumulative percentage mortality of tilapia occurred in a dose-dependent manner when i.p. inoculated with *S. agalactiae* (Figure 5.6), with the highest bacterial concentration resulting in 100% mortality. There were no mortalities in the control group. Bacteria were successfully recovered and identified as *S. agalactiae* from all moribund or dead fish. No bacteria were recovered from the surviving fish inoculated with 1.89 x 10^7 cfu/100 µl of *S. agalactiae* sampled at day 10 or from control fish.

Moribund and dead fish displayed a range of gross external and internal clinical signs of disease. This included: swimming erratically, bi-lateral exophthalmia, corneal opacity, haemorrhaging on abdomen, darkening of the skin and splenomegaly (Figure 5.7).

Histologically, there was evidence of widespread diffuse necrosis and the presence of large numbers of bacteria in all the tissues examined (Figure 5.8). The presence and location of bacteria was confirmed with tissue Gram stains and immunohistochemistry. The immunohistochemistry showed that there was a lack of specificity with the polyclonal antibody that was used. The bacteria and surrounding areas had a reddened appearance for not only the samples that contained *S. agalactiae* but also the *S. iniae* negative controls (Figure 5.9). Negative controls from healthy fish or *S. agalactiae* infected fish with diluted horse serum substitute showed no red antigens or background staining.

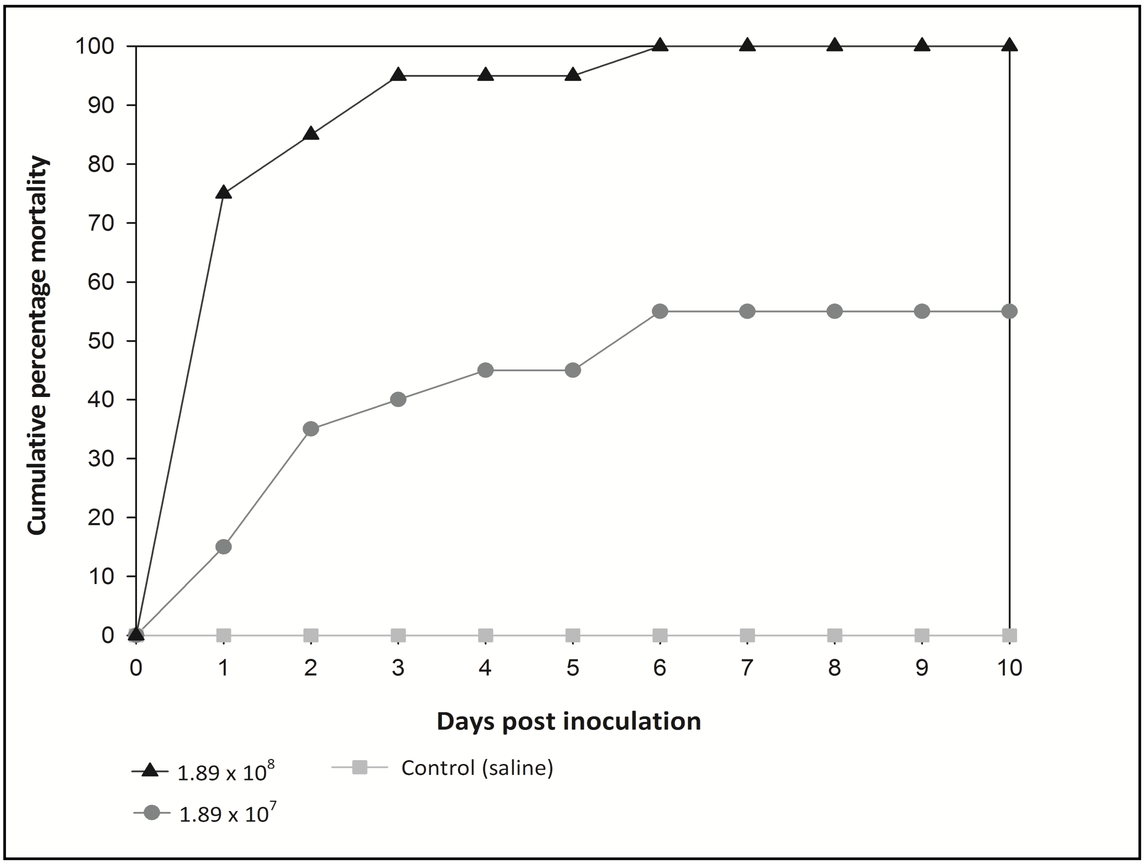

Figure 5.6 The cumulative percentage mortality of tilapia injected intraperitoneally with different *Streptococcus agalactiae* concentrations. Inoculation concentrations are represented as the number of colony forming units of bacteria per inoculum per fish.

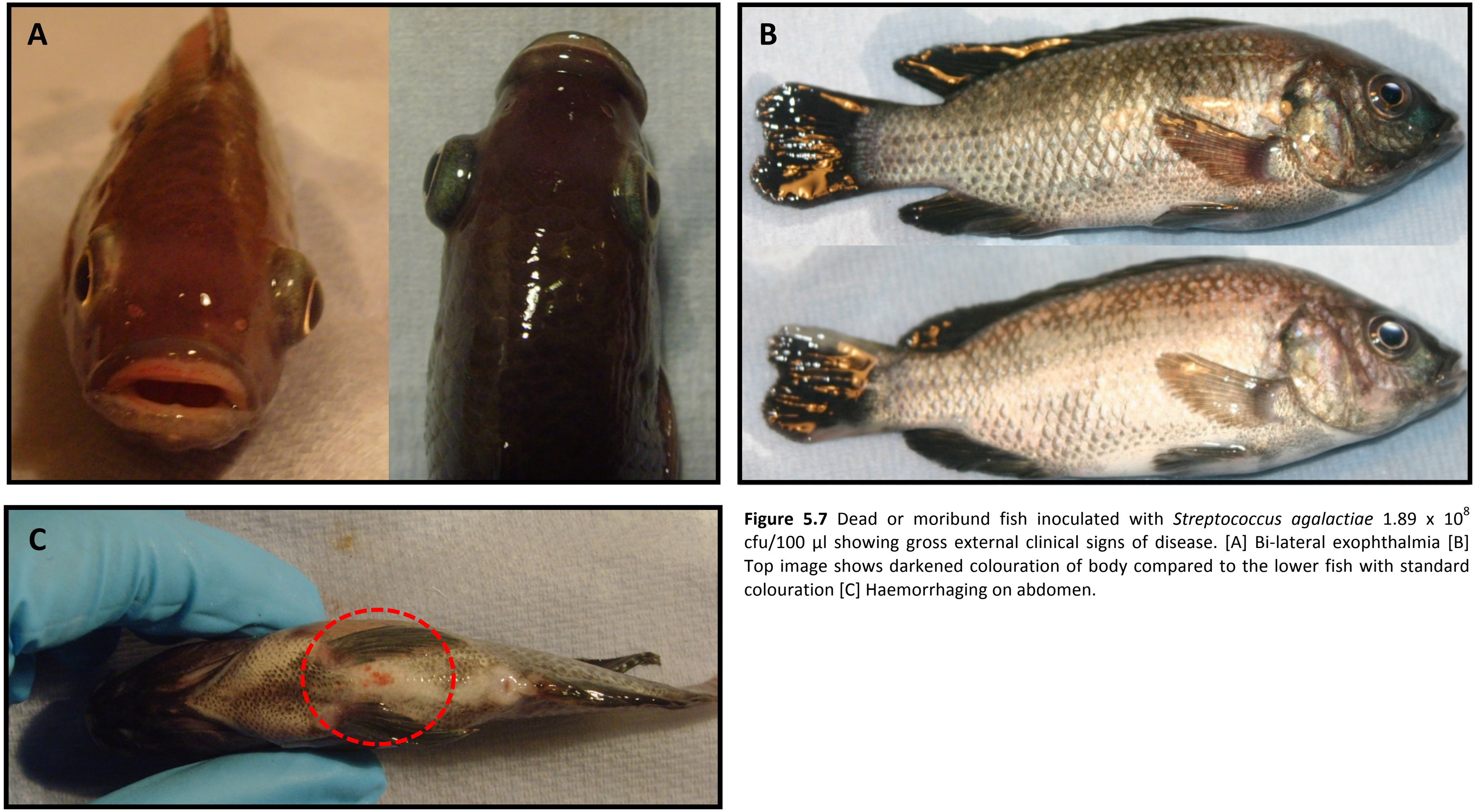

Figure 5.7 Dead or moribund fish inoculated with *Streptococcus agalactiae* 1.89 x 10^8 cfu/100 µl showing gross external clinical signs of disease. [A] Bi-lateral exophthalmia [B] Top image shows darkened colouration of body compared to the lower fish with standard colouration [C] Haemorrhaging on abdomen.

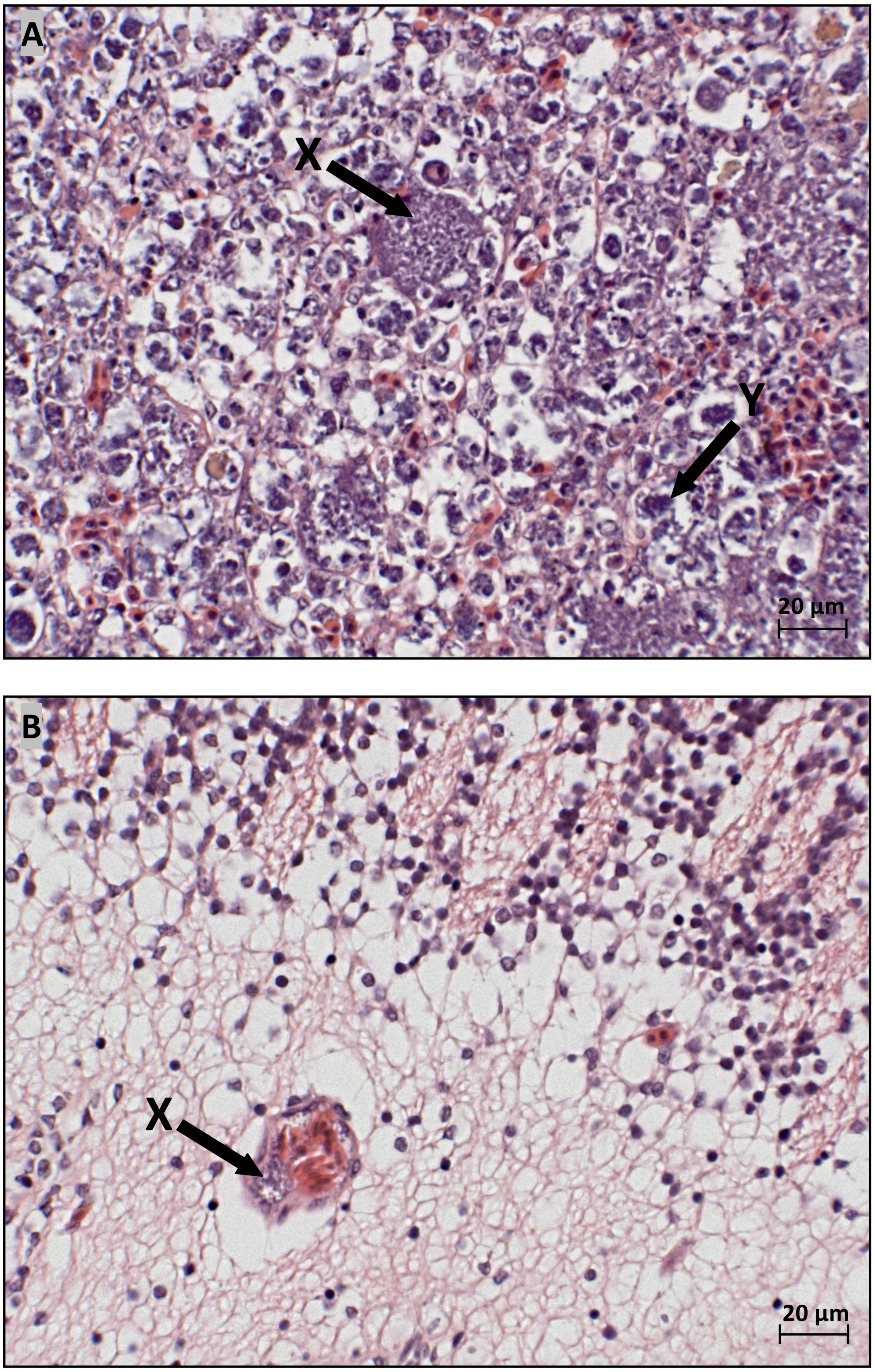

Figure 5.8 Histopathological changes in tilapia experimentally infected with *Streptococcus agalactiae* (H&E). [A] The spleen showing signs of extensive acute necrosis and large accumulations of bacteria throughout the tissue, in blood vessels (X) and apparently intracellular (Y). [B] The brain with accumulations of bacteria throughout in blood vessels (X).

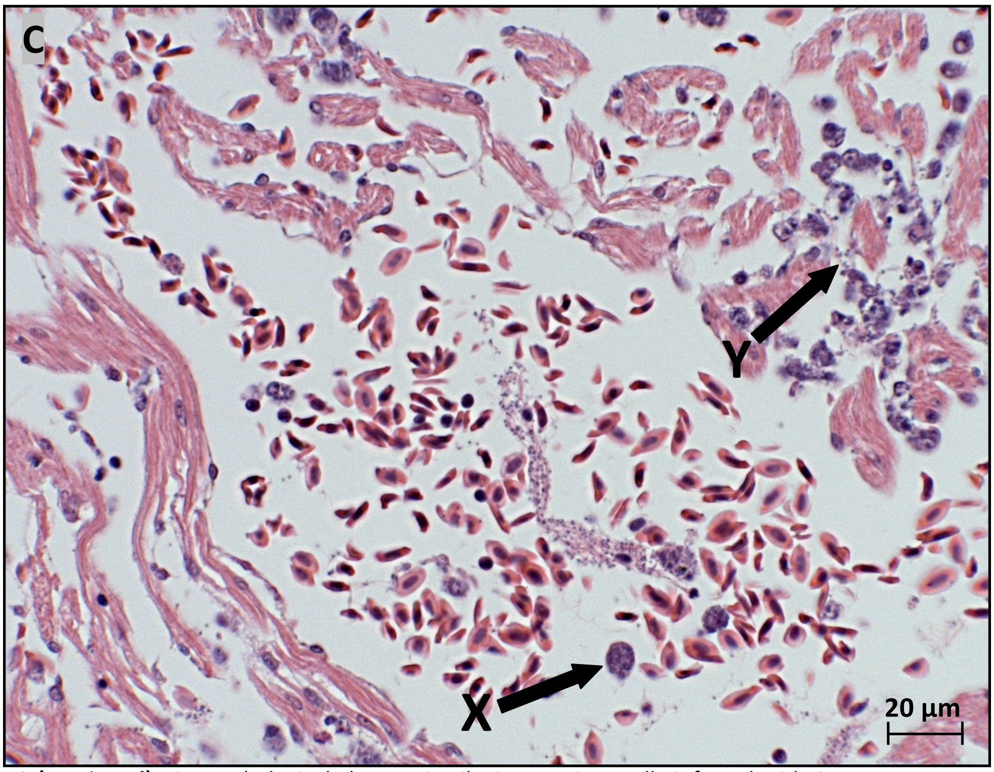

Figure 5.8 (continued) Histopathological changes in tilapia experimentally infected with *Streptococcus agalactiae* (H&E). [C] The heart with evidence of intracellular (X) and extracellular bacteria (Y).

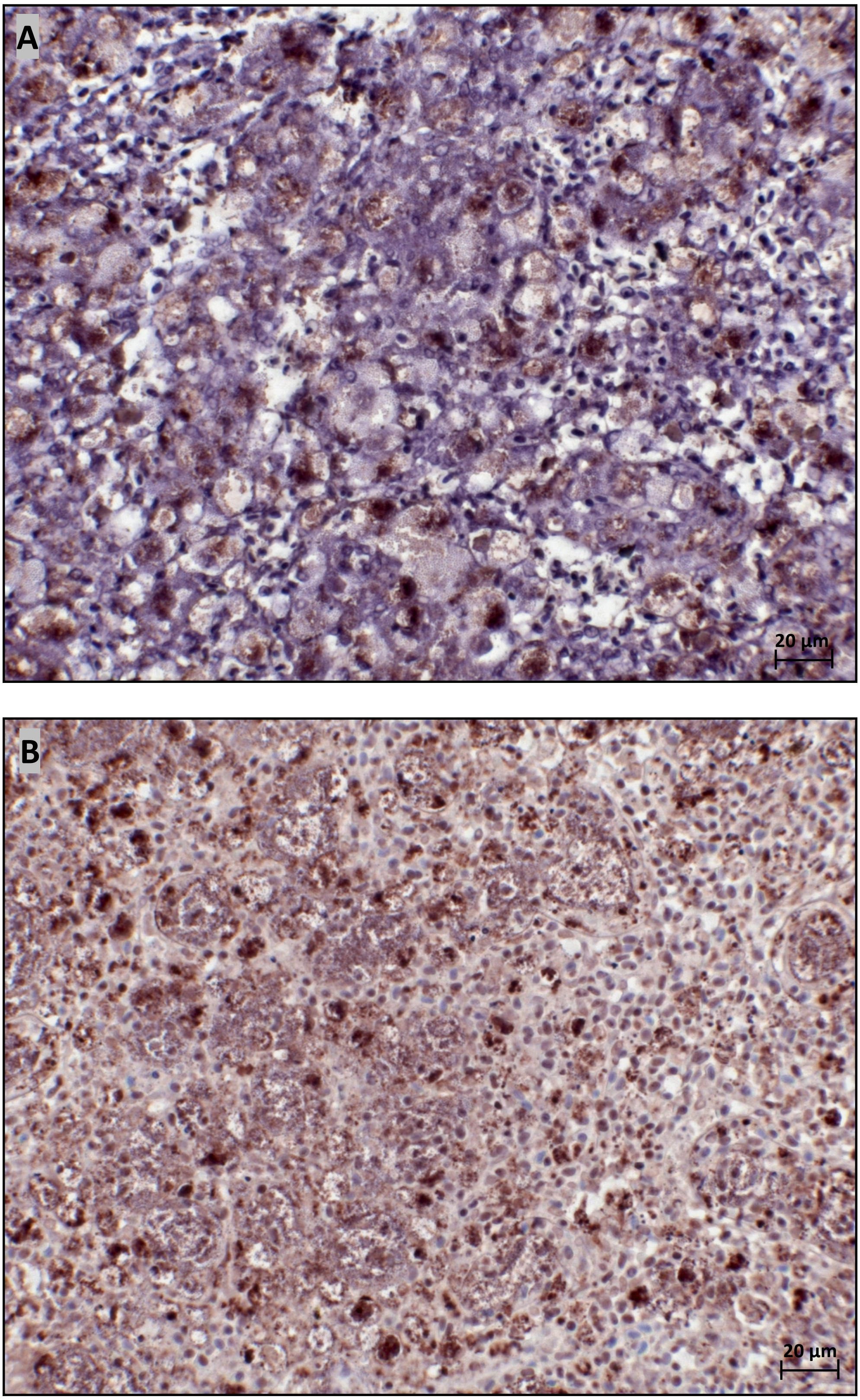

Figure 5.9 Immunohistochemistry in the spleen of tilapia using primary rabbit anti-*Streptococcus agalactiae* polyclonal antibody. [A] Fish from this study experimentally infected with *S. agalactiae* [B] Fish from this study experimentally infected with *S. agalactiae* and *S. iniae* combined. For both [A] and [B] due to the large amount of bacteria present in the tissue the majority of the sections present postive staining assocaited with bacterial antigens.

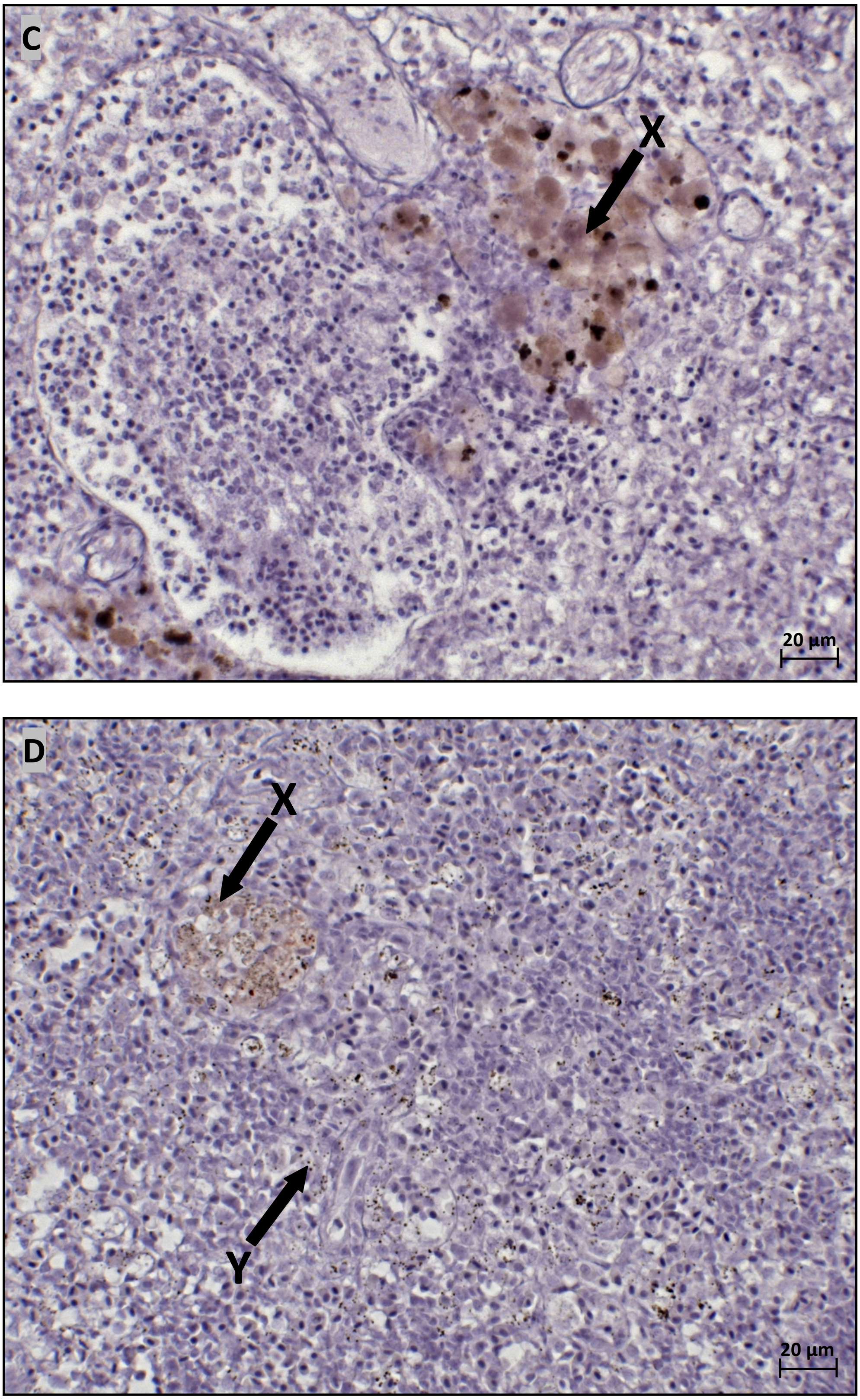

Figure 5.9 (continued) Immunohistochemistry in the spleen of tilapia using primary rabbit anti-*Streptococcus agalactiae* polyclonal antibody. [C] Fish from this study experimentally infected with *S. iniae*. There are areas of postive staining assocaited with bacterial antigens (X) but the majority of bacteria present with no staining. [D] Fish (species unknown) from a clinical case naturally infected with *S. iniae*. There are areas of postive staining assocaited with bacterial antigens (X) but membrane bound melanin granules have also been stained (Y).

5.3.3 Simultaneous *Streptococcus agalactiae* and *Streptoccocus iniae* challenge model

The cumulative percentage mortality of fish receiving *S. agalactiae* and *S. iniae* was 60%. Bacteria were recovered from dead or moribund fish (Figure 5.10) and grew as small white round colonies, however, these colonies were not identified. No bacteria were recovered from the surviving fish when sampled at day 10. The only clinical signs of disease that were apparent during the study included fish swimming erratically and splenomegaly.

In this combined *S. agalactiae* and *S. iniae* challenge the histopathology appearance was indistinguishable from the *S. agalactiae* challenge. There was evidence of widespread diffuse necrosis and the presence of large number of bacteria in all the tissues examined (Figure 5.11). The presence and location of bacteria was confirmed with tissue Gram stains but no immunohistochemistry analysis was performed due to the lack of specificity shown in the primary rabbit anti-*S. agalactiae* polyclonal antibody.

The duplex PCR successfully identify both *S. agalactiae* and *S. iniae* in two kidney samples but for the remaining two samples only *S. agalactiae* was identified. There was banding for both *S. agalactiae* and *S. iniae* type strains at the expected molecular weight and no bands were apparent for the negative control.

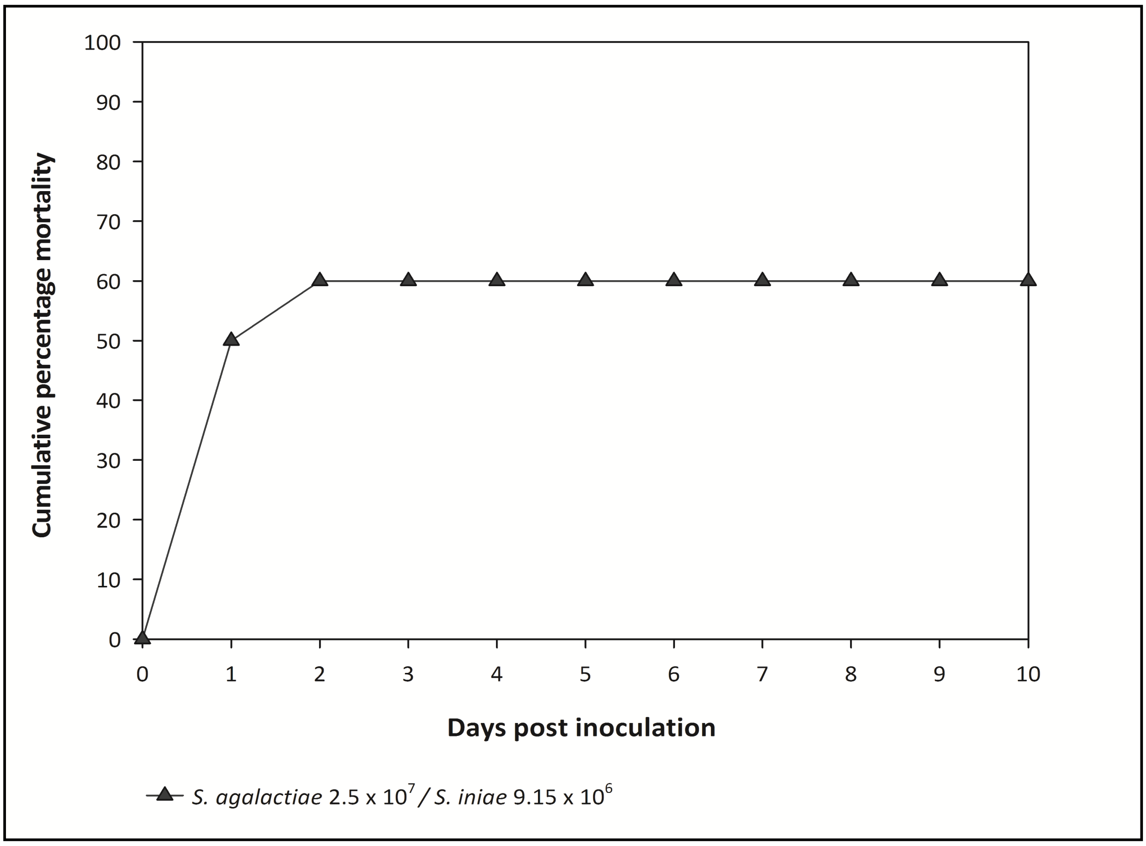

Figure 5.10 The cumulative percentage mortality of tilapia injected intraperitoneally with a combined *Streptococcus agalactiae* and *Streptococcus iniae* suspension. Inoculation concentration is represented as the number of colony forming units of bacteria per inoculum per fish. The bacterial concentration is presented as a range based on the viable cell count results.

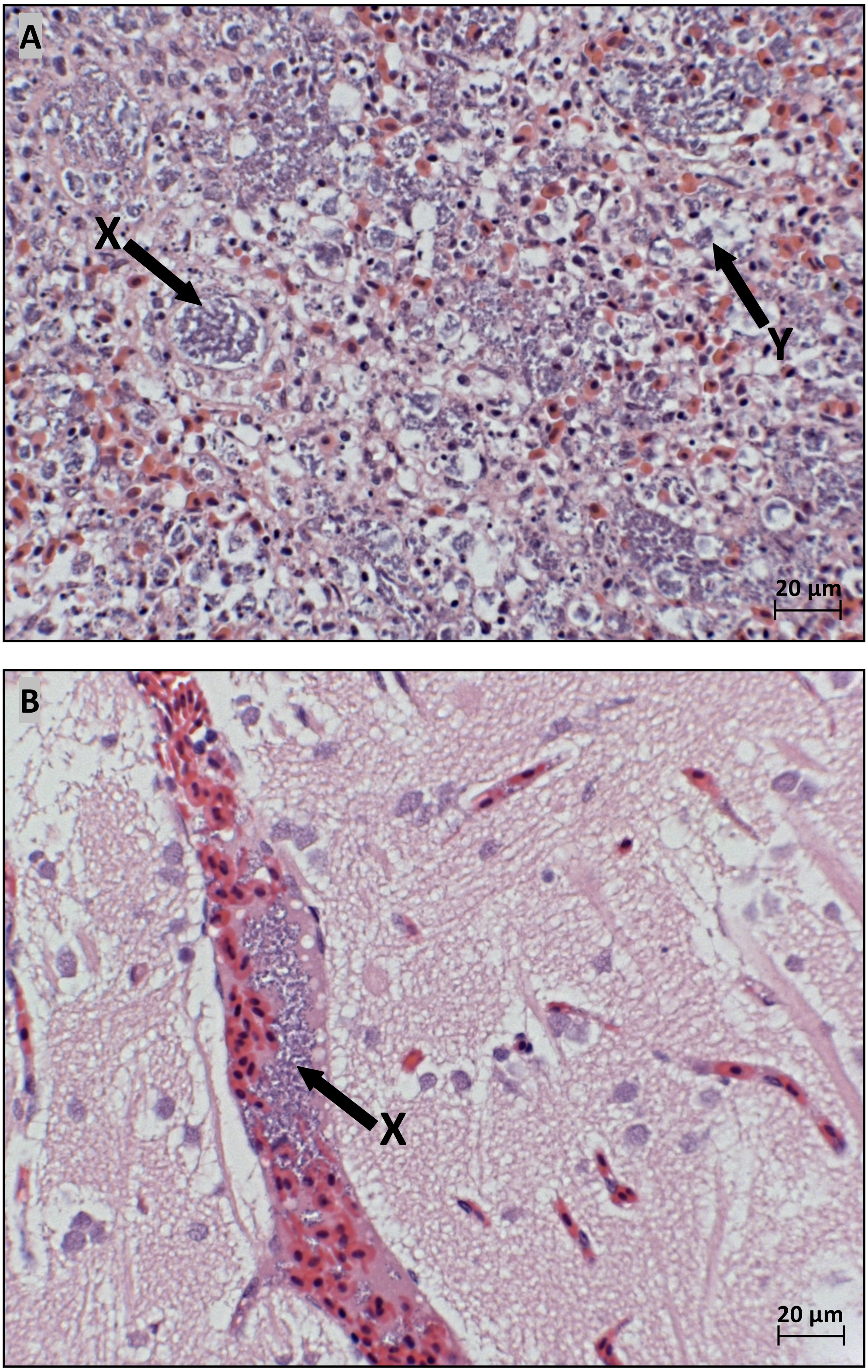

Figure 5.11 Histopathological changes in tilapia experimentally infected with *Streptococcus agalactiae* and *Streptococcus iniae* (H&E). [A] The spleen showing similar appearance to *S. agalactiae* alone with signs of extensive acute necrosis and large accumulations of bacteria throughout the tissue, in blood vessels (X) and apparently intracellular (Y). [B] The brain with accumulations of bacteria throughout in blood vessels (X).

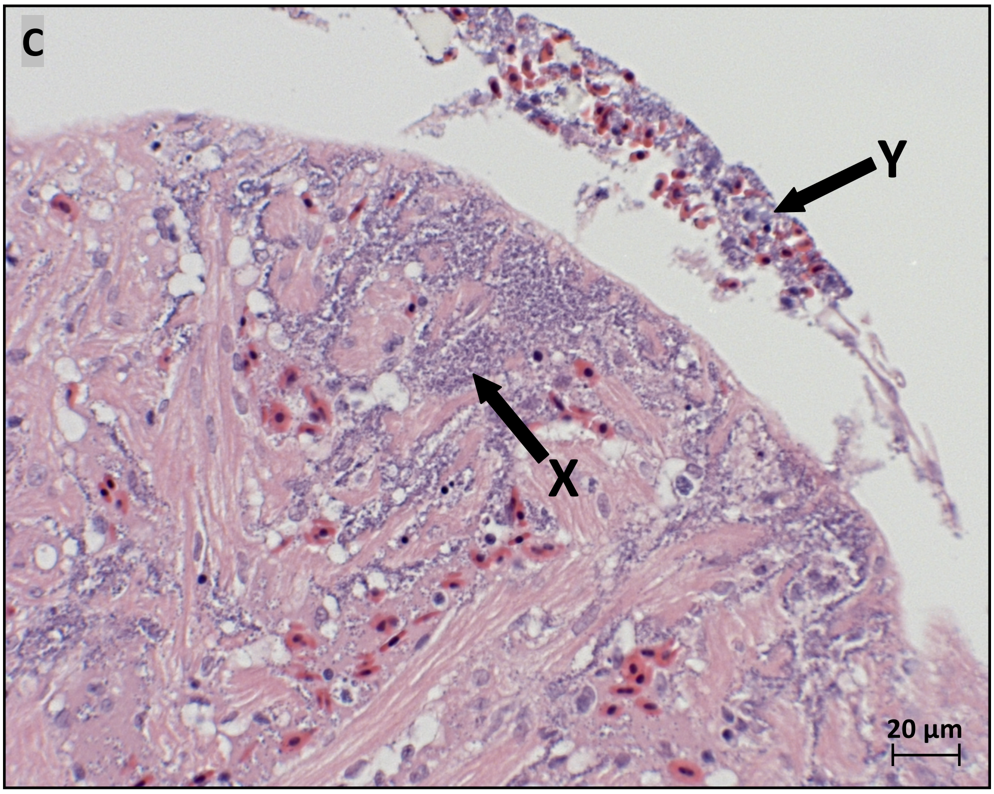

Figure 5.11 (continued) Histopathological changes in tilapia experimentally infected with *Streptococcus agalactiae* and *Streptococcus iniae* (H&E). [C] The heart with areas of massive bacterial accumulation between the myocardial cells (X) and in the pericardium (Y).

5.3.4 VIE tag administration in Nile tilapia

Fish administered the VIE tags showed no adverse behaviour or clinical signs of disease and no mortalities occurred during this study in any of the three tanks. The average amount of feed (± standard deviation) consumed at each feeding period was 3.20 ± 1.79, 3.32 ± 1.65 and 3.59 ± 1.58 for tanks A, B and C respectively. In each treatment group the fish grew in weight ($p < 0.0001$) and length ($p < 0.0001$), however, the VIE treated fish in tank A and tank B were lighter ($p = 0.0374$) but not shorter ($p = 0.9795$) than the non-tagged control fish (Table 5.3). No significance difference was found in fish weight or length between the red and green VIE tagged fish ($p = 0.8992$) and between tanks A and B ($p > 0.999$).

Table 5.3 The mean weight and length of fish (± standard deviation) at various time points post tagging with VIE.

Days post tagging	Tagged fish Tank A	Tagged fish Tank B	Control fish Tank C
	Wet weight (g)		
0	22.97 ± 3.15	22.37 ± 4.05	23.07 ± 4.23
7	27.60 ± 4.61	28.08 ± 5.14	27.06 ± 6.15
14	30.84 ± 5.08	31.06 ± 6.41	32.27 ± 8.60
21	35.68 ± 6.37	36.14 ± 7.75	37.56 ± 11.63
	Length (mm)		
0	86.60 ± 5.74	85.80 ± 6.27	86.00 ± 6.16
7	91.30 ± 3.56	89.90 ± 6.46	88.80 ± 7.04
14	94.00 ± 5.75	95.10 ± 6.92	94.90 ± 9.17
21	99.10 ± 6.66	99.10 ± 7.45	99.20 ± 10.38

Figure 5.12 shows the VIE tags in tilapia 21 days post tagging. There was 100% retention for both red and green VIE tags located at the base of the pectoral fin, in the upper abdomen and in the lower abdomen when observed under UV light 21 days post tagging. Tag visibility was always enhanced using UV light and occasionally made tags visible that would not have been obvious under natural light (Table 5.4 and Figure 5.12). All green VIE tags on the nasal area were retained and visible under both light sources. There were differences in tag visibility between individuals in the two tanks and between the different colour VIE tags as seen in Table 5.4.

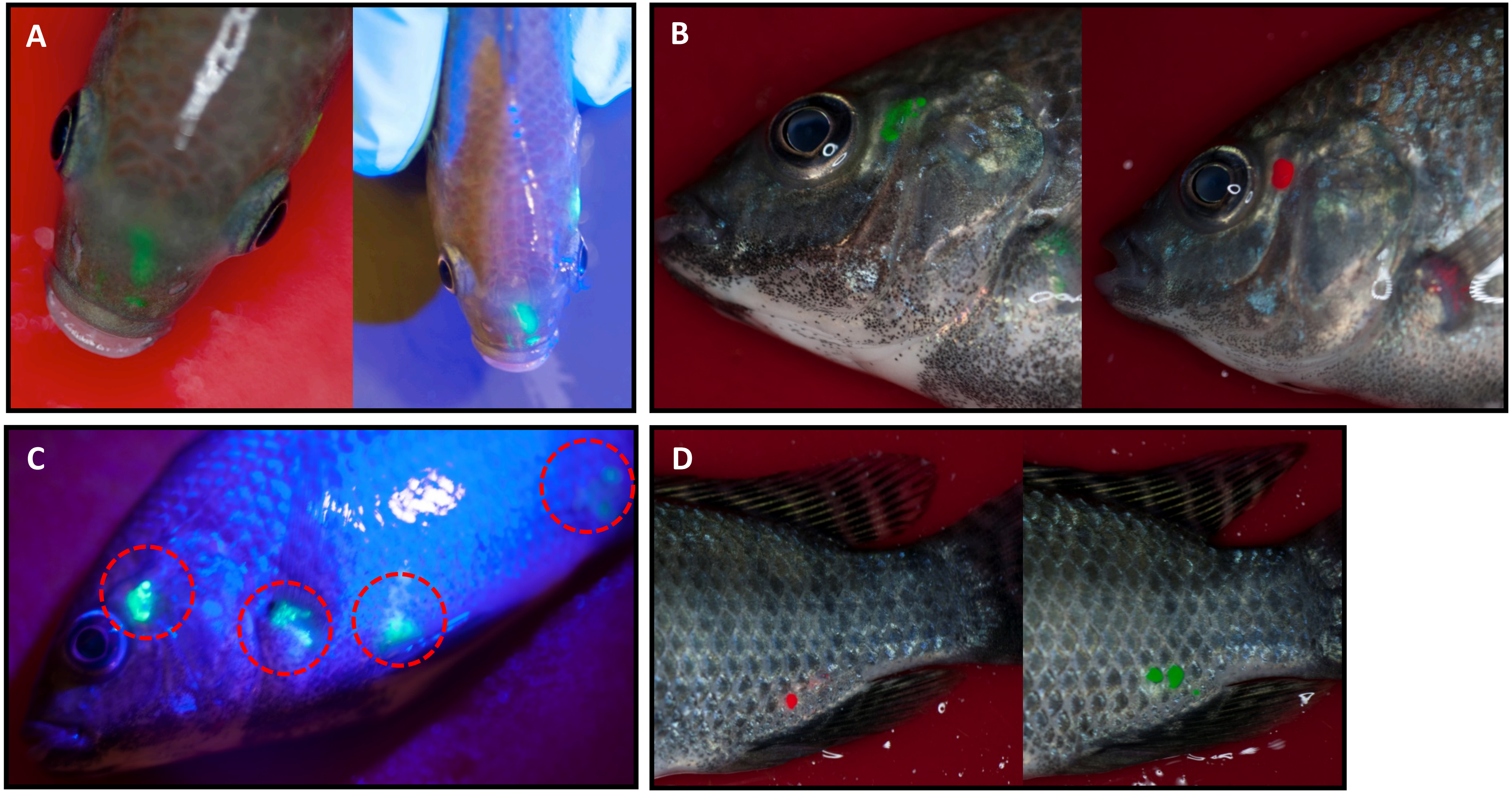

Figure 5.12 Visible implant elastomer tags 21 days post injection into tilapia. [A] Green VIE tag on the nasal and branchiostegal rays inside the operculum under natural light (left image) and UV light (right image) [B] Green and red VIE tags on the branchiostegal rays inside the operculum and at the base of the pectoral fin [C] Green VIE tags observed under UV light [D] Green and red VIE tag on the lower abdomen.

Table 5.4 Total percentage of fish with VIE tag loss 21 days post tagging

Light source	Tank	VIE tag	Tagging area on fish: Nasal (1)	Branchiostegal rays inside the operculum (2)	Base of pectoral fin (3)	Upper abdomen (4)	Lower abdomen (5)
Natural	A	Red	-	60	20	20	-
	B	Red	40	20	-	40	40
	A	Green	-	40	-	-	20
	B	Green	-	-	20	60	40
UV	A	Red	-	40	-	-	-
	B	Red	20	20	-	-	-
	A	Green	-	20	-	-	-
	B	Green	-	-	-	-	-

[-] represents no tag loss in any fish

5.3.5 Application of VIE tags during bacterial challenge

There was a similar pattern of mortality over time between *S. agalactiae* challenged fish with and without VIE tags and between *S. iniae* treatment groups as shown in Figures 5.13 and 5.14. However, a 20% lower mortality was observed in those fish receiving the bacteria and tags and this was regardless of whether fish were tagged 7 days prior to the bacterial challenge or tagged at the same time as the bacterial inoculation. Bacteria were recovered from moribund and dead fish in the *S. agalactiae* challenges were identified as Gram positive cocci, non-motile, oxidase negative and Lancefield Group B positive. From the *S. iniae* challenge bacteria recovered were Gram positive cocci, non-motile, oxidase negative and Lancefield Group B negative.

Tag retention was assessed under natural light when fish were removed from the tank due to morbidity, death or at the end of the 10 day study. It was found that all tags were retained and visible in all fish under natural light.

When the Nikon camera was used in the time-lapse series clear images of the tank and fish were attainable as the time interval that was used was too long. This meant that when a video was made from the time-lapse images it was not possible to monitor the movement of individual fish. On the images that were taken the VIE tags were visible (Figure 5.15) yet when fish were resting at the bottom of the tank and faced away from the camera it was impossible to see these tags. Furthermore, not all the fish were captured in the images that were taken as some fish were out of the field of view.

The time-interval that was used for the GoPro Hero camera provided a video that allowed the individual fish to be monitored. Yet due to a technical fault only 48 hours of video was produced. The images that were produced using the GoPro Hero camera were of a poorer quality compared with the Nikon camera images.

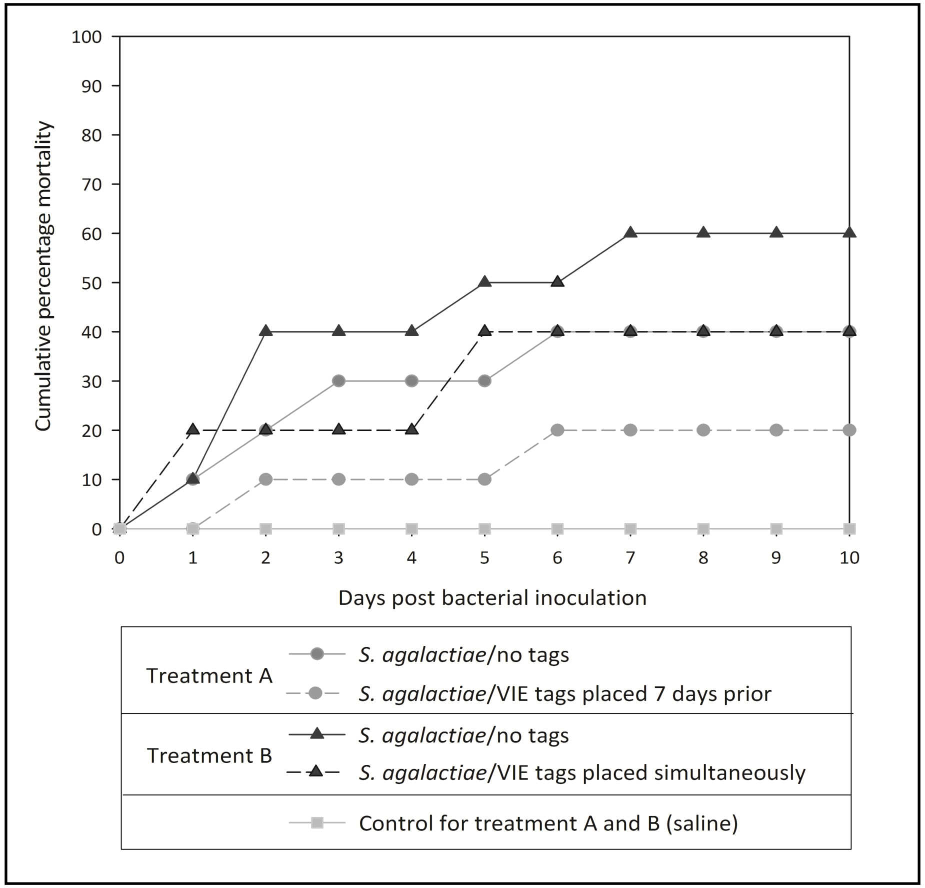

Figure 5.13 The cumulative percentage mortality of fish challenged with *Streptococcus agalactiae* 2 x 10^7 cfu/100 µl. In treatment A tagged fish were injected with VIE 7 days prior to bacterial challenge. In treatment B tagged fish were injected with VIE at same time as the bacterial suspension was administered.

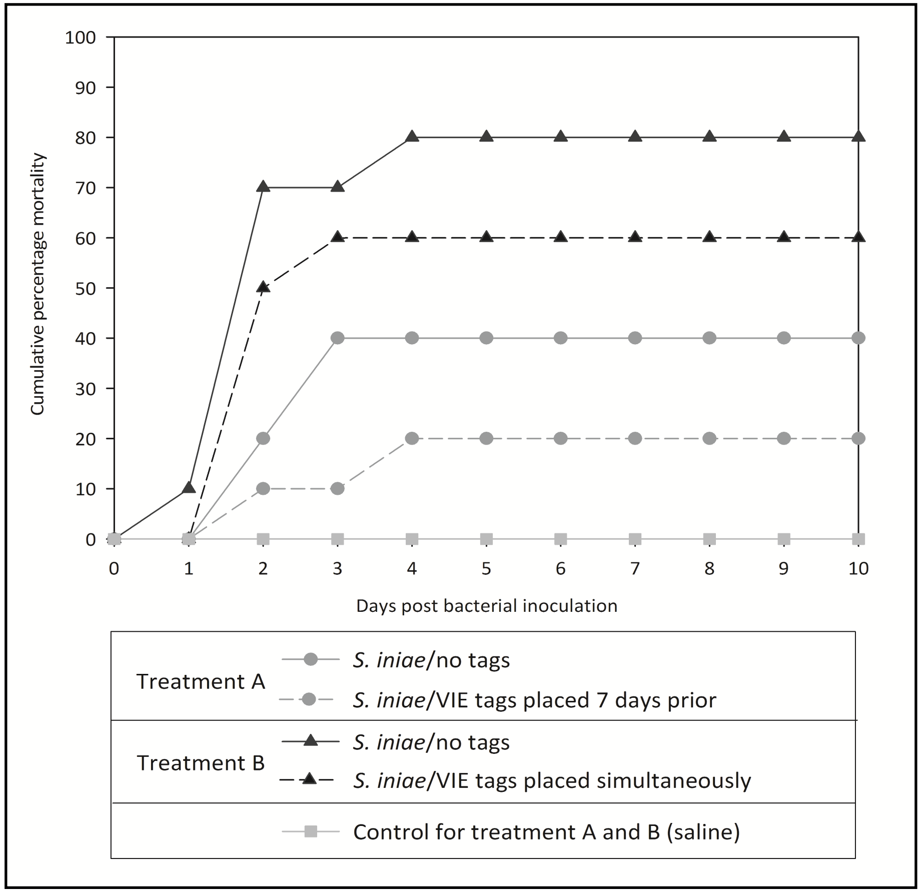

Figure 5.14 The cumulative percentage mortality of fish challenged with *Streptococcus iniae* 1 x 10^7 cfu/ 100 µl. In treatment A tagged fish were injected with VIE 7 days prior to bacterial challenge. In treatment B tagged fish were injected with VIE at same time as the bacterial suspension was administered.

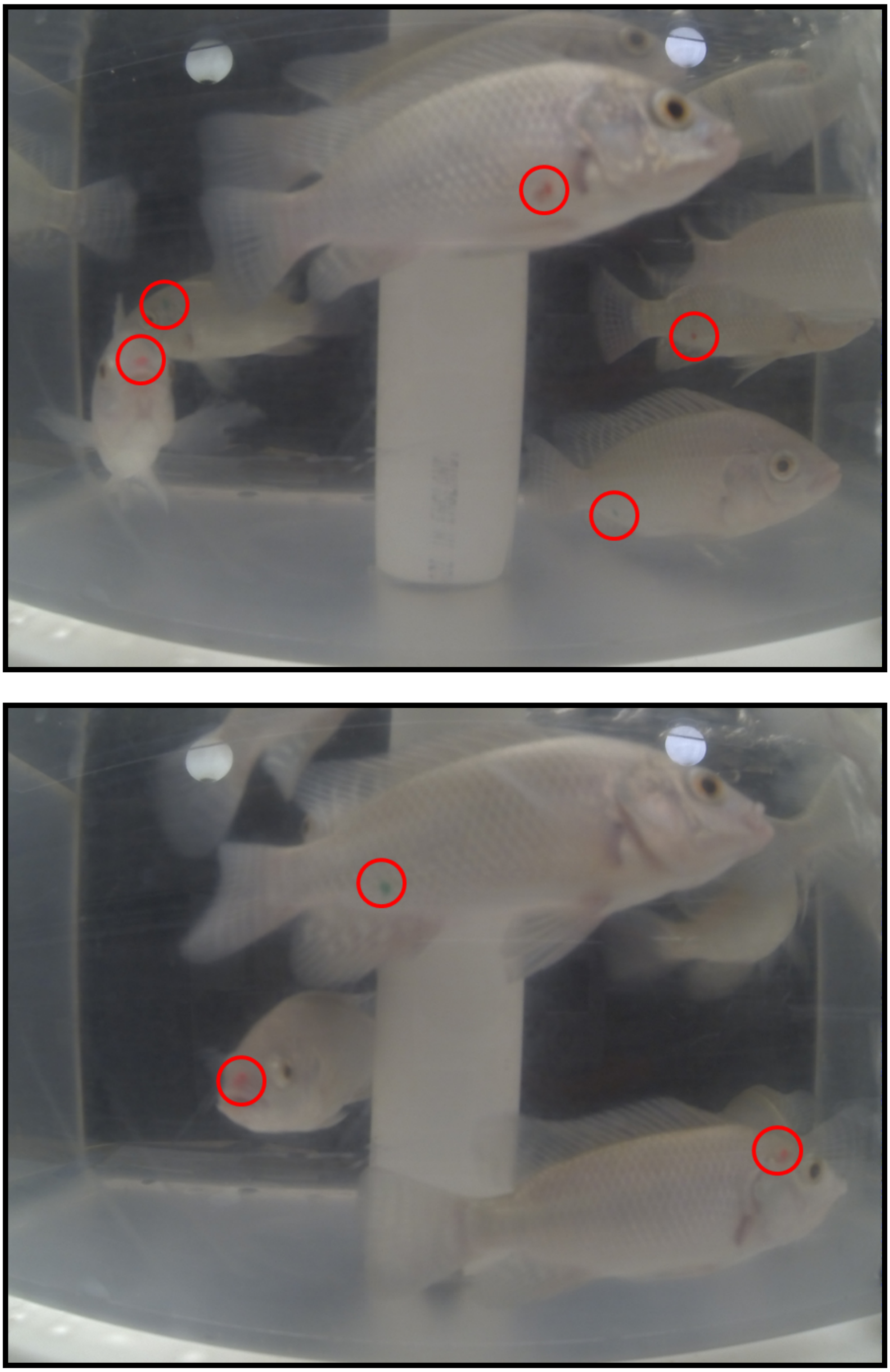

Figure 5.15 Images from a time-lapse series using a Nikon Camera showing the VIE tags on tilapia. VIE tags are highlighted in a red circle.

5.4 Discussion

Experimental challenge models for *S. agalactiae* and *S. iniae* have been successfully achieved through i.p. injection previously (Abuseliana *et al*., 2010; Bromage and Owens, 2002; Perera *et al*., 1997 and Wongsathein, 2012). However, establishing a reproducible challenge model is difficult due to the number of variables that need to be controlled. This also makes comparisons with other research groups difficult. Some of these variables include: the bacterial strain, the fish species from which bacteria was isolated, bacterial dose, route of infection and the weight and age of the fish (Evans *et al*., 2002; Pretto-Giordano *et al*., 2010)

In this study, when tilapia were challenged with *S. iniae* the total mortality rates occurred in a dose dependent manner. However the inoculum concentrations used either gave a low level of mortality or caused all the fish to die. Therefore it was theorized that a dose of 1 x 10^7 cfu/100 µl would provide approximately 50% mortality. Indeed, when this dose was used in first VIE tagging trials the total mortality rate was 40%. There were fewer inoculum challenges used in the *S. agalactiae* challenge due to fish supply difficulties. Fortunately, the inoculum concentrations used produced tilapia morbidity/mortality rates in a dose dependent response and one of the inoculums gave a mortality rate of 55%. Morbidity/mortality rates of approximately 50% were desirable because it was unknown what the effect of a combined inoculum would have on the mortality rate. Since the concentration that caused this approximate 50% mortality was relative similar, 2 x 10^7 cfu/100 µl for *S. agalactiae* and 1 x 10^7 cfu/100 µl for *S. iniae,* it was theorized that a total bacterial of 1.5 x 10^7 cfu/100 µl would be suitable for the combined bacterial challenge. Unfortunately, due to a lack of available fish, only one bacterial concentration could be tried and there was no control group for the combined *S. agalactiae/S. iniae* challenge. Caution must consequently be used when drawing conclusions from this challenge. Nevertheless, since clinical signs of disease were apparent and PCR analysis showed that the two bacterial species were detected in the kidney of some of these fish it is reasonable to assume that fish morbidity and/or mortality was a direct result of *S. agalactiae* and *S. iniae.*

The clinical signs of disease and general histological findings observed were similar to previous reports (Baums *et al*., 2013; Chen *et al*., 2007; Delannoy, 2013; Wongsathein, 2012). Additionally, work from Delannoy (2013) and Wongsathein (2012) was conducted in the same facilities that was used in this study and all the fish were sourced from the same aquarium. This highlights the reproducibility of the *S. agalactiae* challenge model.

It was observed that there was a considerably higher bacterial load in the organs of fish infected with *S. agalactiae* compared with *S. iniae*. In addition, *S. agalactiae* was detected in the organs such as the brain and heart whereas in the *S. iniae* challenge, no bacteria was identified in these organs. These findings are in agreement with Chen *et al*. (2007) who conducted a comparative histopathology study of *S. iniae* and *S. agalactiae* infected tilapia. Their study also found a high bacterial load in tissues and in the circulation of *S. agalactiae* infected fish, but not in *S. iniae* infected fish. Additionally, Chen *et al.* (2007) also found the bacterial cells of *S. iniae* were rarely observed in the internal organs.

Unfortunately, the immunohistochemistry results were of limited value in the study presented. The primary rabbit anti-*S. agalactiae* polyclonal antibody should react with the type specific carbohydrate of Group B *Streptococcus,* but this was not supported in the study presented. Indeed, results from Delannoy (2013) using the same polycloncal antibody successfully highlighted *S. agalactiae* antigens from infected tilapia tissue. However, in this study the antibody showed a lack of specificity as both *S. iniae* controls also showed reddening around bacteria indicating a positive immunohistochemistry result. It is possible that the (control) clinical case tissue that was used could have been naturally infected with both *S. agalactiae* and *S. iniae* and this was not detected during identification proceedings. In addition, membrane bound melanin granules were positively stained in the tissue sections investigated thus further complicating the immunohistochemistry analysis. However, samples were also used from the *S. iniae* challenge in this study where there was no concern of contamination with *S. agalactiae*. This was not totally unexpected; polycloncal antibodies generally have a lack of specificity as there is an increased chance of cross-reactivity with similar antigens (Bromage, 2004). There were fewer controls used when assessing the anti-*Streptococcus iniae* monoclonal antibody. There were no clinical cases available at the time and the *S. agalactiae* challenge had not yet been performed at this stage. However, this became irrelevant when the rabbit anti-*S. agalactiae* polyclonal antibody showed a lack of specificity. Two robust immunohistochemistry techniques were required for both *S. agalactiae* and *S. iniae* if they were to be of use in assessing the combined *S. agalactiae* and *S. iniae* challenge.

The aim of this study was to collect information to help design a sequential bacterial challenge study to investigate the pathogenesis of *S. agalactiae* and *S. iniae* in more detail. The results of the studies performed provided the bacterial concentrations to be used and determined that histopathology and PCR analysis would be suitable methods to identify and assess *S. agalactiae* and *S. iniae* in infected tilapia tissue. For a sequential challenge model, fish need to be sampled at certain time points. VIE tagging was used so that through time-lapse photography the time at which the first and last fish showed signs of morbidity after exposure to bacteria could be established. This would help identify key stages in disease progression and provide a rough approximation to the time fish are exposed to bacteria are infected, show signs of morbidity/mortality or are in recovery.

Before this novel application of VIE tags could be trialed the suitability of using VIE tagging in tilapia had to be assessed. The results of this study did demonstrated the potential of using VIE for tagging tilapia as they were retained and visible when administered in several locations over the fish. It was subsequently shown that ten tilapia within a tank could be individually identified by their VIE tag thus supporting the potential of using VIE for tagging tilapia for observational purposes and thus its suitability in experimental research studies.

The ability to see the tags once introduced into the fish is commonly affected by the colour of the VIE (Soula *et al*. 2012). In this study both green and red VIE tags were tested, where the green was more noticeable than red in the tilapia. This is probably species specific as the varied and sometimes dark pigmentation often found in tilapia resulted in the green tag being more easily detected than the red. Other colours are available but in this study both red and green VIE tags were visible and recognizable when fish were in or out of water. Clear visibility is a requisite of any tagging method and the results of this study found that although the VIE tag could be seen with the naked eye, the visibility was enhanced using a UV light which is in agreement with FitzGerald *et al*. (2004) and Simon and Dörner (2011). Lack of visibility is thought to be due to the depth of the tag in the fish (Zeller and Cairns 2010) or tags being forced into deeper tissues regions and skin pigmentation increasing whilst the fish grows (Simon and Dörner 2011). The first VIE experiment performed in this study was conducted over 21 days and whilst the fish did grow during this time it is more plausible that any impairment in tag visibility was due to the lack of tagging experience resulting in the VIE tags being injected deeper into tissue than required. This is supported by the fact that tag visibility was already reduced 7 days post tagging by which time the fish had not grown significantly. Additionally, all tags were retained and visible without the aid of UV illumination during the subsequent 10 day pathogenesis studies when tagging experience had been gained.

One of the more surprising results from this study was the lower mortality rate observed in the VIE tagged fish compared with non-tagged fish during the bacterial challenges. It may be that the VIE tags invoked an acute immune response in the fish or indirectly effected growth and immunomodulation through behavioural changes. Such changes could either be in the tagged fish or the reaction of their conspecifics to the tags. In this study fish were exposed to VIE tags either at the same time [B1 – 5] or 7 days prior [A1 – 5] to receiving the bacteria. There were higher mortality rates in treatment groups B1 – 5 than those in treatment groups A1 – 5. The bacterium recovered from dead fish in treatment groups A1 – 5 were used to make the bacterial inoculum used in the treatment groups B1 – 5. The bacterium used in treatments B1 – 5 is therefore considered to have been passaged, which can potentiate bacteral virulence. This was unfortunate but did not account for the 20% mortality reduction in the tagged fish compared to non-tagged fish. Results would suggest that the lower mortality observed was due to the tag but the reason is unknown. Certainly, others have used visible alphanumeric tags (Lin *et al*. 2006) or latex tags (Alcorn *et al*. 2005) for vaccination studies but did not report any immunomodulation effect. Frederick (1997) previously described VIE tags as being relatively compatible with the physiology of marked fish and supported this by citing unpublished research which found that histology samples taken from tagged rainbow trout did not show any cellular changes typical of inflammation or tag rejection. Hohn and Petri-Hanson (2013) also stated that the VIE is a non-immunogenic polymer but no further details were provided. The effect of VIE on fish immune response remains uncertain. Further work is required to assess any acute humoral or cellular immune responses caused by VIE tagging.

VIE tagging has shown to have no effect on the survival and growth (weight and length) of various other fish species held in the laboratory under controlled conditions. These species including the European eel (*Anguilla anguilla*) (Simon and Dörner 2011), pinfish (*Lagodon rhomboides*) (Matechik *et al*., 2013), rainbow trout (*Oncorhynchus mykiss*) (Leblanc and Noakes 2012), pikeperch (*Sander lucioperca*) (Zakęś *et al*., 2013), gilthead seabream (*Sparus auratus*) (Astorga *et al*., 2005) and zebrafish (*Danio rerio*) (Hohn and Petrie-Hanson 2013). However, whilst the VIE tags had no effect on the overall survival of the tilapia in this study when administered without a bacterial challenge, tagging was significantly associated with differences in growth. Hoey and McCormick (2006) did describe higher growth rates in coral reef fish (*Pomacentrus amboinensis*) when marked using an uncured elastomer compared with cured elastomer tags or unmarked fish, however, this was not statistically significant. In general others using VIE tags in fish have not reported an effect of growth even when their investigations were conducted over a longer time period, up to 6 months. In both the study presented here and the work described by Hoey and McCormick (2006) small sample sizes were used, and to confirm any effect on growth using VIE tags additional studies should be conducted using larger samples sizes. This would improve confidence in the results.

For monitoring the fish during the study, a 24 hour light regime was essential for the time-lapse photography. Before the study was initiated the fish were kept in a 12 hour light: 12 hour dark cycle. This change in photoperiod is considered to have no impact on tilapia growth as a study by Elsbaay (2013) showed that a change in photoperiod does not affect the growth of tilapia up to 30 days. Although the fish used in Elsbaay's study were of a smaller weight (5 g initially) the relatively short time frame in which pathogenesis studies are conducted indicates that the use of a 24 hour light regime is acceptable. This would not be the case for longer studies as there would be a concern about the effect of photoperiod on fish growth, metabolism and possibly immune functions. Nevertheless, a more suitable method for monitoring the fish during a bacterial challenge needs to be developed as the approaches tried in this study had considerable drawbacks.

VIE tags were found to be suitable in several tag locations on tilapia and both elastomer colours were noticeable. This provides the capacity to individually identify up to five fish per colour during a bacterial challenge or other experiments. The coding capacity could be enhanced by utilising the additional elastomer colours available. Although retention rates were generally high in the VIE tagged fish tagging experience is valuable to maximise tag retention and visibility under natural light. Overall, VIE tagging is a suitable marking method in tilapia and could be a novel approach to identify individual animals within a treatment group.

The work presented clearly showed that VIE tagging could be applied in aquatic microbial pathogenesis studies.

Although the time-lapse photography was not successful in this study it was apparent that fish infected with *S. iniae* experienced an acute infection with morbidity/mortality occurring 1 – 3 days after exposure. Whereas, in the *S. agalactiae* challenge, fish showed a more chronic infection with mortalities occurring from 1 – 6 days after exposure. This indicates for the sequential bacterial challenge that *S. iniae* requires more time points shortly after exposure whereas for *S. agalactiae* the time points need to be extended to cover a longer time period. The results from this study will help formulate a robust sequential study to investigate the pathogenesis of *S. agalactiae* and *S. iniae* in tilapia.

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Chapter 6

Sequential *Streptococcus agalactiae and Streptococcus iniae* challenges performed *in vivo* in Nile Tilapia

6.1 Introduction

Streptococcus agalactiae and *S. iniae* are invasive bacterial pathogens shown to infect a wide variety of fish species (Baums *et al*., 2013), resulting in high morbidity and mortality during disease outbreaks. In China, for example, large-scale streptococcal outbreaks resulted in a loss of approximately US$0.4 billion in 2011 (Chen *et al*., 2012). Several experimental challenge models and pathological studies have been produced for both *S. agalactiae* and *S. iniae* (Baums *et al*., 2013; Wongsathein, 2012, Chen *et al*., 2007). However, to the best of the authors knowledge, there is no published literature on the progression of infection and pathology during a disease outbreak and the level of individual variability within a population. In addition, both *S. agalactiae* and *S. iniae* have been recovered and identified from diseased fish at the same geographical location (Conroy, 2009; Yuasa *et al*., 2008) yet there are no reports that investigate an *S. agalactiae* and *S. iniae* simultaneous infection.

The aims of this study were to investigate the clinical signs, *in vivo* bacterial distribution and histopathological changes that develop during a time course experimental infection of Nile tilapia (*Oreochromis* niloticus) with either [1] *S. agalactiae* [2] *S. iniae* or [3] *S. agalactiae* and *S. iniae* combined. The experimental design for these sequential challenges was based around information obtained from previous pilot challenges (Chapter 5). The results obtained will allow a direct comparison between the infection pathways of *S. agalactiae* and *S. iniae* and show any changes of such pathways during a simultaneous infection.

6.2 Materials and methods

6.2.1 Study design

Sequential study

To study the progression of infection and pathology, six tanks of fish (10 fish per tank) were challenged with either [1] *S. iniae* [2] *S. agalactiae* or [3] *S. agalactiae*/ *S. iniae* combined. One tank of fish was sampled at particular time points, post bacterial exposure (Table 6.1 and Figure 6.1). For sampling, fish were euthanized by anaesthetic overdose of benzocaine solution (Sigma-Aldrich, Buchs, Switzerland) and examined for clinical signs of disease and histopathology samples taken and processed as previously described (Section 5.2.1.5). A small sample (≈ 0.03 g) of kidney, spleen, brain and liver was also taken aseptically and stored at -70 °C until used for bacterial identification purposes. Control tanks were sampled as described above at day 14.

Batch variation

During the *S. iniae* sequential challenge there was no fish morbidity or mortality as expected. The experiment was terminated at day 3 and the challenge repeated. Simultaneously, 20 fish were collected from another batch of fish (Tropical Aquarium, Institute of Aquaculture, University of Stirling) and challenged with *S. iniae* or *S. agalactiae* (Figure 6.1, Table 6.1). This was to determine if the lack in morbidity observed was due to fish batch variation as this was an unexpected reaction given the previous challenge experiments performed in this study. Conducting these challenges concurrently ensured all other variables such as the bacterial concentration and water temperature were consistent between treatment groups (Table 6.1). Any fish mortalities or morbidities were sampled for bacterial recovery and identification as previously described (Section 5.2.1.4).

6.2.2 Fish

Nile tilapia (*O. niloticus*) were provided from in-house stocks at the Institute of Aquaculture, University of Stirling. Two different populations of mixed sexed fish were used in this study, batch A for experiments 1 – 4 and batch B for experiments 5 and 6. Fish were stocked at 10 fish per tank. The average (± S.D.) weight and water temperature used for each challenge is summarised in Table 6.1. The husbandry conditions were the same as those described in Section 5.2.1.1 with the exception that fish received feed more frequently throughout a day.

Table 6.1 Summary of the fish husbandry conditions for the different sequential bacterial challenges

Challenge number	***S. iniae* [1]** 1	***S. agalactiae*** 2	***S. iniae/S. agalactiae*** 3	***S. iniae* [2]** 4	***S. iniae*** 5	***S. agalactiae*** 6
Dates	1.10.13 – 8.10.13	7.10.13 – 21.10.13	14.10.13 – 28.10.13	14.10.13 – 28.10.13	14.10.13 – 28.10.13	14.10.13 – 28.10.13
Batch of fish	A	A	A	A	B	B
Average fish weight in experimental tanks (± S.D.) (g)	25.32 ± 3.20	24.73 ± 2.8	25.86 ± 3.34	25.21 ± 3.30	24.35 ± 2.90	24.82 ± 3.05
Average fish weight in control tanks (± S.D.) (g)	33.30 ± 1.82	32.23 ± 1.73	31.75 ± 1.35		n/a	n/a
Inoculum concentration (cfu/100 µl)	9.9×10^6	2.4×10^7	*S. agalactiae*: 2.4×10^7 *S. iniae*: 6.6×10^6	6.6×10^6	6.6×10^6	2.4×10^7
Average water temperature (°C)	25.32 ± 3.20	24.90 ± 0.23	24.78 ± 0.26	24.78 ± 0.26	24.78 ± 0.26	24.78 ± 0.26
Total number of fish	70[1]	90[1]	70[1]	60	10	10
Sample time points for bacterially challenge fish[2]	6h, 12h, day 1, day 2, day 3 and day 14	6h, 12h, day 1, day 2, day 3, day 5, day 7 and day 14	6h, 12h, day 1, day 2, day 3 and day 14	6h, 12h, day 1, day 2, day 3 and day 14	n/a	n/a

[1] This includes the control tank (n = 10)
[2] Time (hours and days) after the bacterial inoculation was administered that one tank of fish was sampled
[n/a] not applicable, not available

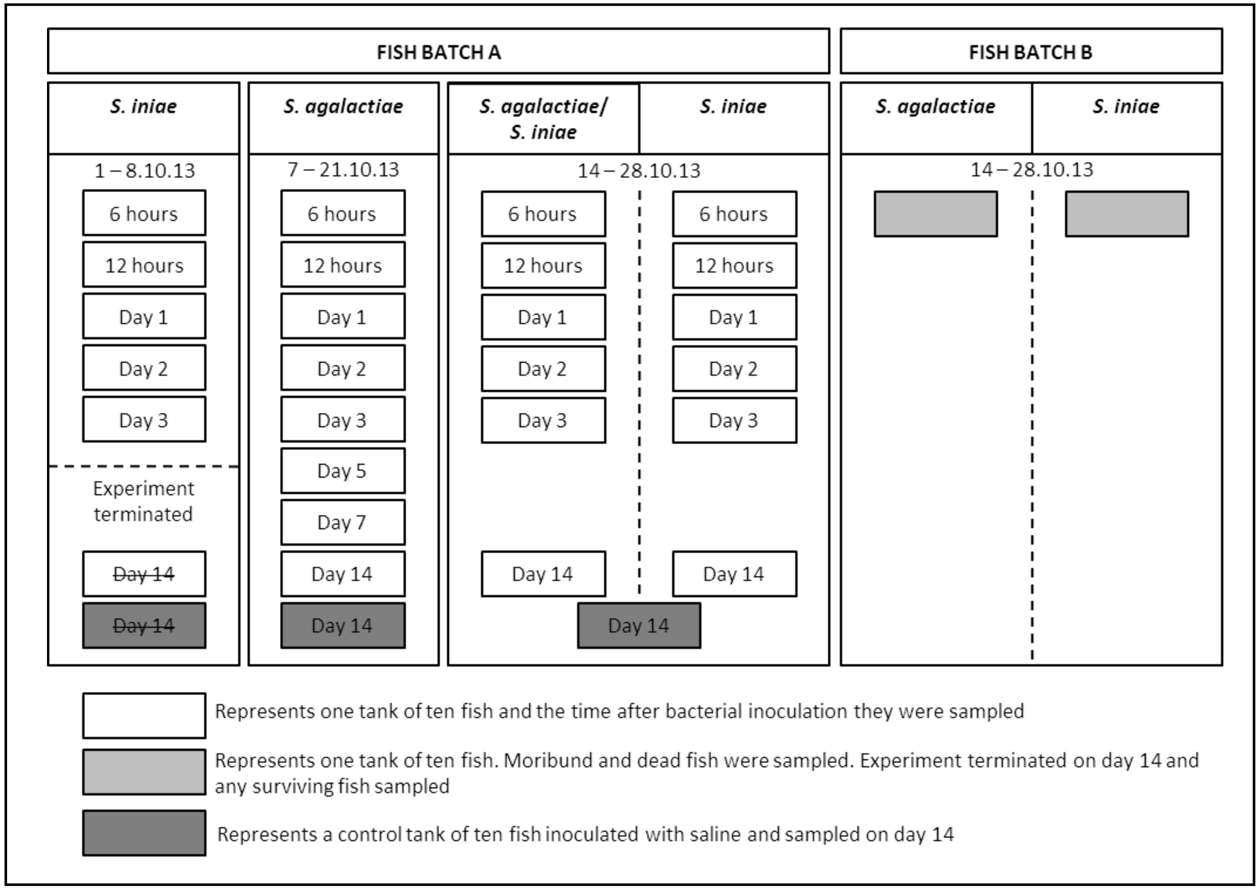

Figure 6.1 Schematic illustration of the experimental design for the sequential challenges of *Streptococcus agalactiae* and *Streptococcus iniae*

6.2.3 Preparation and inoculation of bacterial suspensions

Passaged isolates of *S. agalactiae* B and *S. iniae* C, stored on frozen cryobeads, were prepared as previously described (Section 5.2.1.2). For the bacterial challenges the bacterial concentrations used were [1] *S. iniae* 1 x 10^7 cfu/100 µl [2] *S. agalactiae* 2 x 10^7 cfu/100 µl and [3] *S. agalactiae* and *S. iniae* combined: *S. iniae* 5 x 10^6 cfu/50 µl and *S. agalactiae* 1 x 10^7 cfu/50 µl. A viable cell count was carried out to determine the bacterial concentration as described by Miles and Misra (1938) and in Section 3.2.4. Fish were inoculated as described previously (Section 5.2.1.3). Control groups were i.p. injected with 0.85% saline (100 µl/ fish). Some challenges were performed concurrently to one another and in such circumstances only one control tank was used (Figure 6.1).

6.2.4 Bacterial identification from infected fish tissue

DNA extraction was performed on the tissue samples either using RealPure DNA extraction kit (Thistle Scientific) or the SSTNE/salt precipitation method. The RealPure DNA extraction was conducted according to manufacturer's instructions; the SSTNE/salt precipitation was based on the method described in Pardo *et al*. (2005). Briefly, tissue samples were homogenized in SSTNE extraction buffer (See appendix), SDS (10%) and proteinase K (10 mg/ ml). The samples were then incubated overnight at 55 °C. RNAase (2 mg/ml) was further added before an incubation at 37 °C for 1 hour. The DNA was purified in accordance with Pardo *et al*. (2005).

A duplex PCR was performed as described previously (Section 2.2.6.2). In addition to the duplex PCR, a nested PCR was also used based on the protocol described by Ferguson *et al.* (2010). Briefly, to make a 25 µl reaction mixture there was a single illustra PuReTaq Ready-To-Go PCR Bead (GE Healthcare, Buckinghamshire, UK), 1 µl of each primer 20F and 1500R (Table 6.2), 1 µl of infected tissue DNA extract and MilliQ water to volume. For the primary

PCR, the thermocycling parameters were: an initial preheating cycle at 95 °C for 4 minutes followed by 25 cycles of 95 °C for 1 minute, 55°C for 1 minute, 72°C for 90 seconds, and a final step of 72°C for 5 minutes. These PCR products produced were then used in a duplex PCR as described previously (Section 2.2.6.2).

Table 6.2 The oligonucleotide primers used in a nested PCR for the identification of *Streptococcus agalactiae* or *Streptococcus iniae* from infected tilapia tissue

Primer	Direction	Nucleotide sequence (5' – 3')	Target gene	Target region (bp)	Pathogen	Reference
20F 1500R	Forward Reverse	AGAGTTTGATCATGGCTCAG GGTTACCTTGTTACGACTT	16S rRNA	≈ 1,500	Most eubacteria	Ferguson *et al.*, 2010

6.3 Results

6.3.1 Batch variation

There were differences between the total level of morbidity and/or mortality between the different batches of fish (Table 6.3). In particular, there was a considerable difference in the total level of mortality between the *S. iniae* challenges; when fish batch A was used there was no mortality or noticeable morbidity whereas there was a 100% mortality in fish batch B. Fish batch A was used in the sequential challenges and subsequently the total mortality and/or mortality rate ranged from only 0 – 10%.

Table 6.3 The total percentage mortality at the time when each tank was sampled. The day at which morbidity or mortality was observed is indicated in brackets.

Time after inoculation tank sampled	Fish batch A			Fish batch B	
	S. agalactiae/ S. iniae	***S. iniae***	***S. agalactiae***	***S. iniae***	***S. agalactiae***
6 hours	0	0	0	-	-
12 hours	0	0	0	-	-
Day 1	0	0	10 (Day 1)	-	-
Day 2	0	0	0	-	-
Day 3	0	0	0	-	-
Day 5	-	-	10 (Day 5)	-	-
Day 7	-	-	10(Day 6)	-	-
Day 14	**10 (Day 7)**	**0**	**10 (Day 6)**	**100 (Day 1,2,3,4)**	**20 (Day 1 and 6)**

6.3.2 Bacterial identification from fish tissue

Two DNA extraction methods were used on spleen and kidney samples taken from fish 6 hours or 24 hours after inoculation with either *S. agalactiae* or *S. iniae*. The subsequent DNA samples produced were used in a duplex and nested PCR. *Streptococcus iniae* could not be detected using either DNA extraction or PCR methods. Whereas, *S. agalactiae* was successfully identified in samples using both DNA extraction and PCR methods. There was no apparent difference between the DNA extraction methods on PCR amplification however there was an overall higher level of DNA extracted (ng/µl) when the SSTNE/salt precipitation method was used rather than the RealPure DNA extraction kit. The nested PCR increased band intensity of

S. agalactiae samples during gel electrophoresis, compared with the duplex PCR alone, however the number of positive results between the two PCR methods were the same

6.3.3 Sequential challenges

During the sequential *S. iniae* challenge there were no mortalities or noticeable morbidity in any of the tanks. There were also very few clinical signs (Table 6.4); however, haemorrhagic intestinal content was found in several fish from 6 hours up to 3 days post inoculation (Figure 6.2). During histopathological analysis there were only a few changes; the brain showed signs of inflammation 6 hours post inoculation and the spleen showed necrosis and vacuolation 6 – 24 hours post inoculation. This contrasted dramatically with batch B where there was a 100% mortality, bacteria were present in all organs examined and there was acute septicaemia 24 hours post inoculation.

Table 6.4 Summary of the clinical signs of disease and other observations noted at the time of sampling. Numbers represent the total percentage of fish within the tank presenting with clinical signs. [-] represents that the clinical sign was not apparent in any of the sampled fish.

Bacterial challenge	Sampling time point	Clinical signs of disease														Other	
		Enlarged spleen	Haemorrhagic intestinal content	Enlarged kidney	Haemorrhaging externally around fins and/or abdomen	Reddened liver	Lesion on heart	Pop-eye	Enlarged heart	Corneal opacity	Discoloured liver	Swollen abdomen	Fluid behind eye	Darkening of body	Lesions on fins/abdomen	Abdominal fat	Lesions on jaw
S. iniae	6 hours	-	30	-	-	-	-	-	-	-	-	-	-	-	-	20	-
	12 hours	-	60	-	-	-	-	-	-	-	-	10	-	-	-	-	-
	Day 1	10	-	-	10	-	-	-	-	-	-	-	-	-	-	60	-
	Day 2	-	30	-	-	-	-	-	-	-	-	-	-	-	-	30	10
	Day 3	-	10	-	-	-	-	-	-	-	-	-	-	-	-	50	-
	Day 14	-	-	-	-	-	-	-	-	-	-	-	-	-	40	-	-
S. agalactiae	6 hours	10	20	30	20	-	-	-	10	-	-	-	-	-	-	-	-
	12 hours	40	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Day 1	20	-	-	10	-	-	-	-	-	-	-	-	-	-	-	-
	Day 2	40	10	20	10	-	-	-	-	-	-	-	10	-	-	-	-
	Day 3	70	30	10	-	10	20	-	-	-	-	-	-	-	-	-	-
	Day 5	60	10	10	-	20	20	-	-	-	-	-	-	-	-	-	-
	Day 7	30	-	-	-	-	10	-	10	10	30	-	-	-	40	-	-
	Day 14	-	33	-	-	-	-	-	-	-	-	-	-	30	40	100	10
	CONTROL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	67	17
S. agalactiae/ *S. iniae*	6 hours	10	60	-	10	-	-	-	-	-	-	-	-	-	-	-	-
	12 hours	-	30	20	-	20	-	-	-	-	-	-	-	-	10	-	-
	Day 1	40	-	-	-	-	-	40	-	-	-	-	-	-	-	60	-
	Day 2	40	20	10	10	10	-	10	-	-	-	-	-	-	-	90	10
	Day 3	60	30	10	-	30	-	10	-	-	-	-	-	-	30	90	-
	Day 14	-	22	-	-	-	-	-	-	-	22	-	-	-	44	67	22
	CONTROL	-	-	-	-	-	-	-	-	-	-	-	-	-	33	83	-

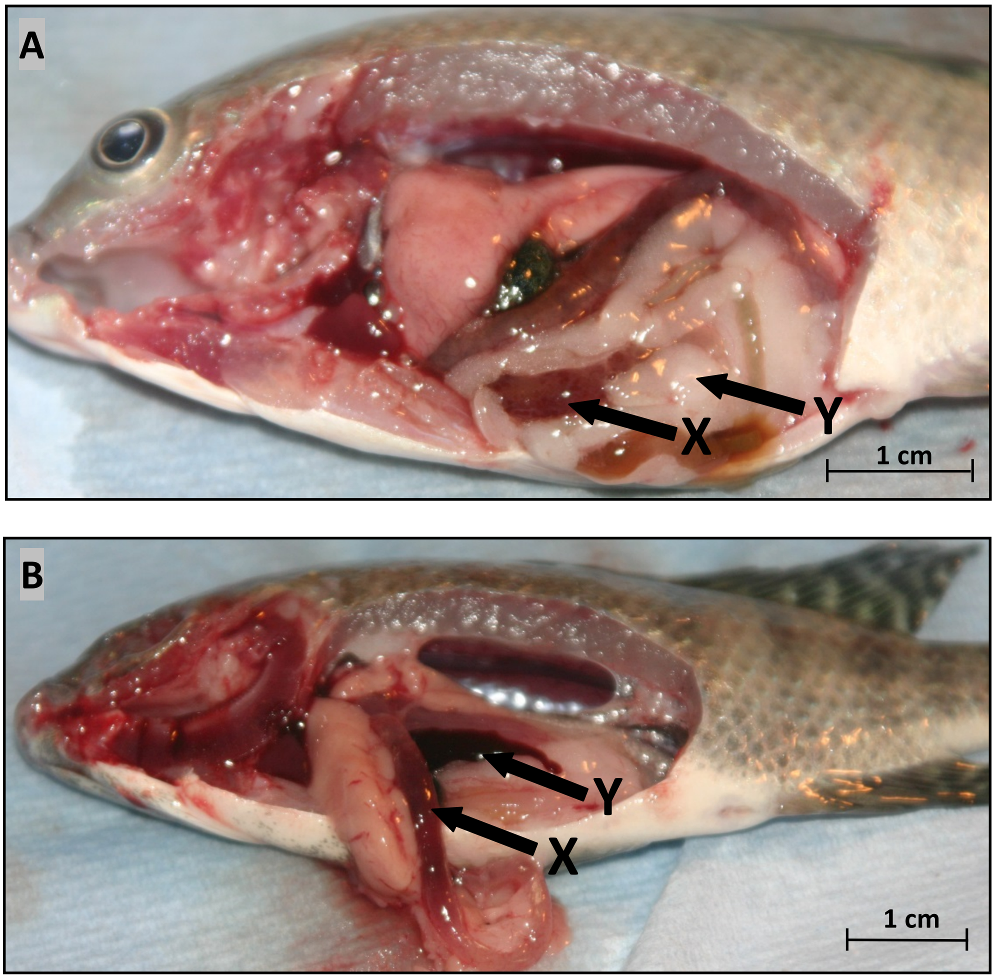

Figure 6.2 Clinical signs of disease and other observation from fish experimentally infected with *Streptococcus iniae*. [A] Fish sampled 6 hours post inoculation with haemorrhagic intestinal content (X) and abdominal fat (Y). [B] Fish sampled 2 days post inoculation with haemorrhagic intestinal content (X) and an enlarged spleen (Y).

Clinical signs and histopathology changes were apparent in *S. agalactiae* infected fish just 6 – 12 hours post infection (Table 6.4). The brain showed signs of inflammation, there were early signs of epicarditis (Figure 6.3) and there was separation of the epithelium in gill lamelli. Individual bacterial were detected in the spleen 6 hours post inoculation, the heart, liver, spleen and kidney 24 hours post inoculation and by 72 hours post inoculation bacteria were noticeable in all organs. Through sequential sampling the progression of disease was detected as shown in Figure 6.3; just 6 hours post inoculation the heart from infected tilapia showed early signs of epicarditis, by 24 hours there was evidence of more severe inflammation and by 72 hours the pathology has progressed to extensive septic pericarditis with septic vegetative valvular endocarditis and large accumulations of bacteria.

The histopathology analysis from fish mortalities that occurred during the *S. agalactiae* sequential challenge and those from fish batch B (Table 6.3) were similar. There was evidence of widespread diffuse necrosis and the presence of large number of bacteria in all the tissues examined. Similarly, fish that survived the *S. agalactiae* inoculation but were sampled 14 days later from batch A and batch B had similar histopathology findings. The brain had eosinophilic granular cells (EGC) and there was melanomacrophages in the kidney and spleen.

After 6 – 12 hours post inoculation with *S. agalactiae* fish were shown to have enlarged spleens, haemorrhaging around fins, haemorrhagic intestinal content (Figure 6.4) and long trailing faecal casts. Lesions were seen externally, on fins and on abdomen (day 7 and 14), and internally on the heart (day 3 – 7) (Figure 6.4). Fish sampled 1 – 7 days post inoculation showed a variety of different clinical signs of disease (Table 6.4) however by day 14 there was considerably fewer clinical signs apparent.

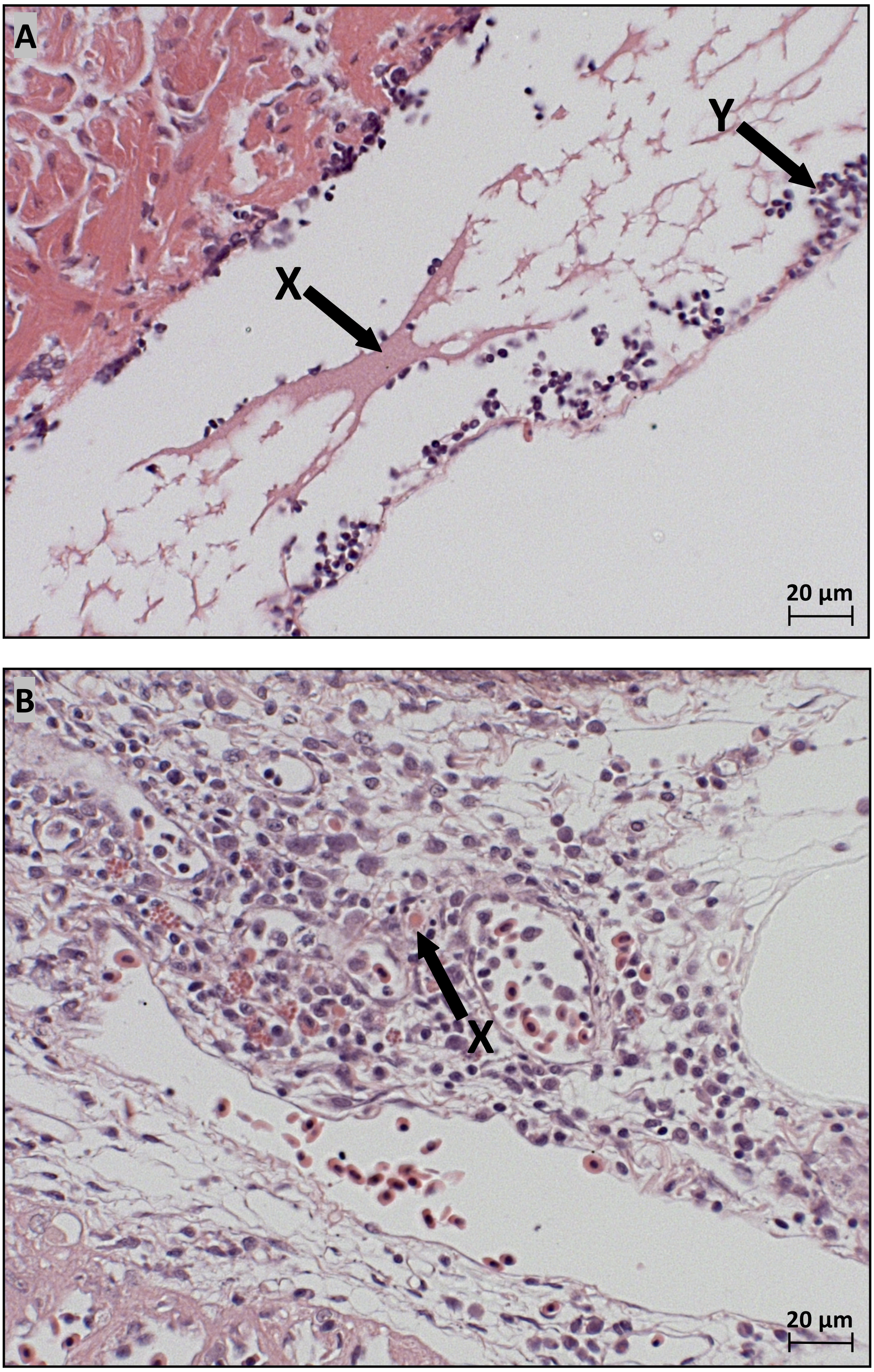

Figure 6.3 The heart from a tilapia experimentally infected with *Streptococcus agalactiae* (H&E). [A] 6 hours post challenge there is early signs of epicarditis with fibrinous deposits (X) and accumulation of inflammatory cells (Y). [B] 24 hour post challenge there is evidence of more severe inflammation and some bacteria can be seen (X).

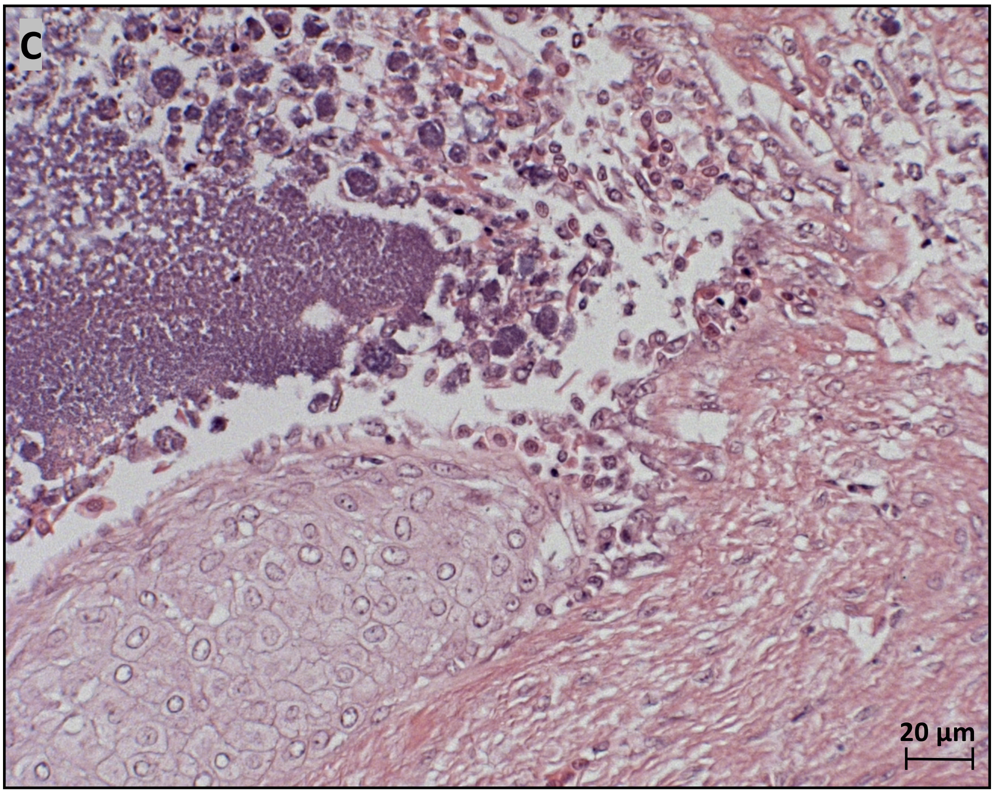

Figure 6.3 continued The heart from a tilapia experimentally infected with *Streptococcus agalactiae* (H&E). [C] 72 hours post challenge the pathology has progressed to extensive septic pericarditis with septic vegetative valvular endocarditis and large accumulations of bacteria (H&E).

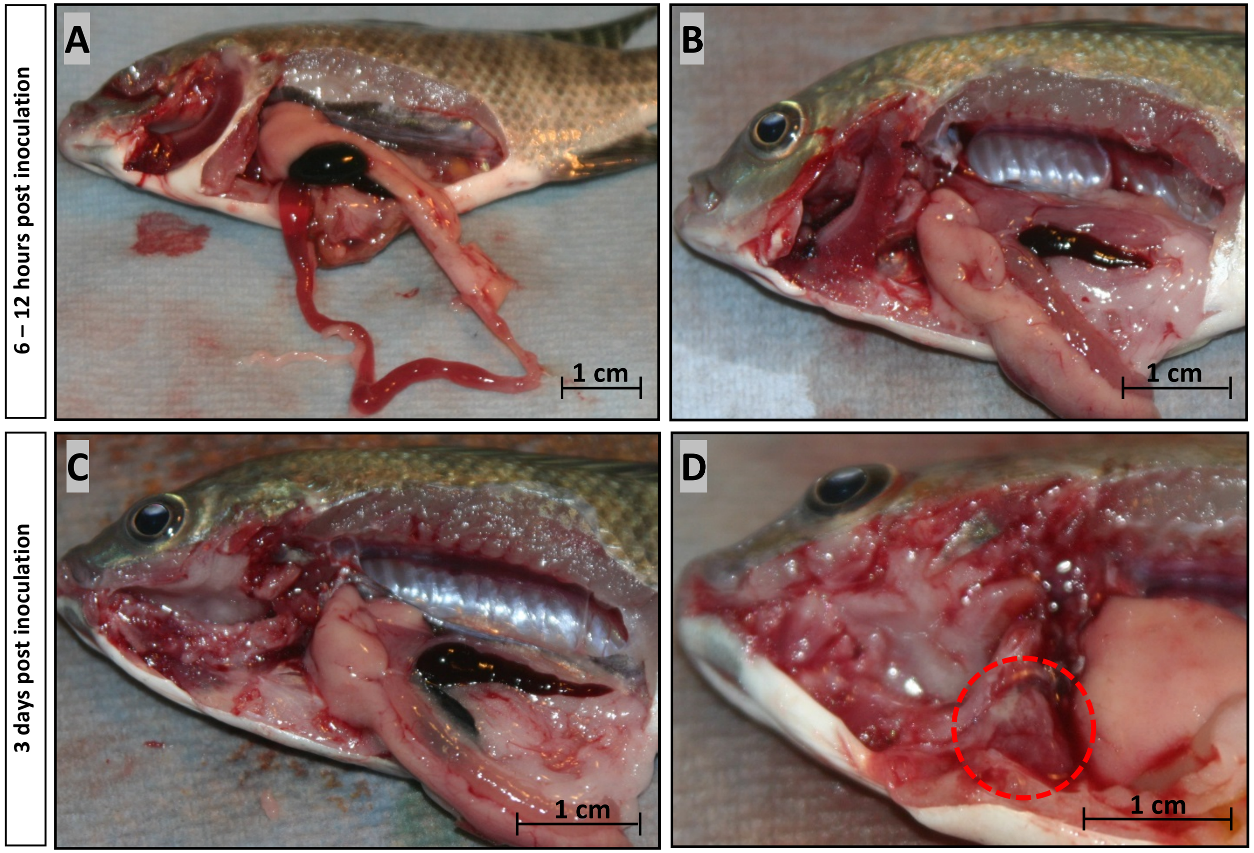

Figure 6.4 Clinical signs of disease and other observation from fish experimentally infected with *Streptococcus agalactiae.* [A] Fish sampled 6 hours post inoculation with haemorrhagic intestinal content [B] Fish sampled 6 hours post inoculation with an enlarged spleen [C] Fish sampled 3 days post inoculation with an enlarged spleen [D] Fish sampled 3 days post inoculation with lesions on the heart.

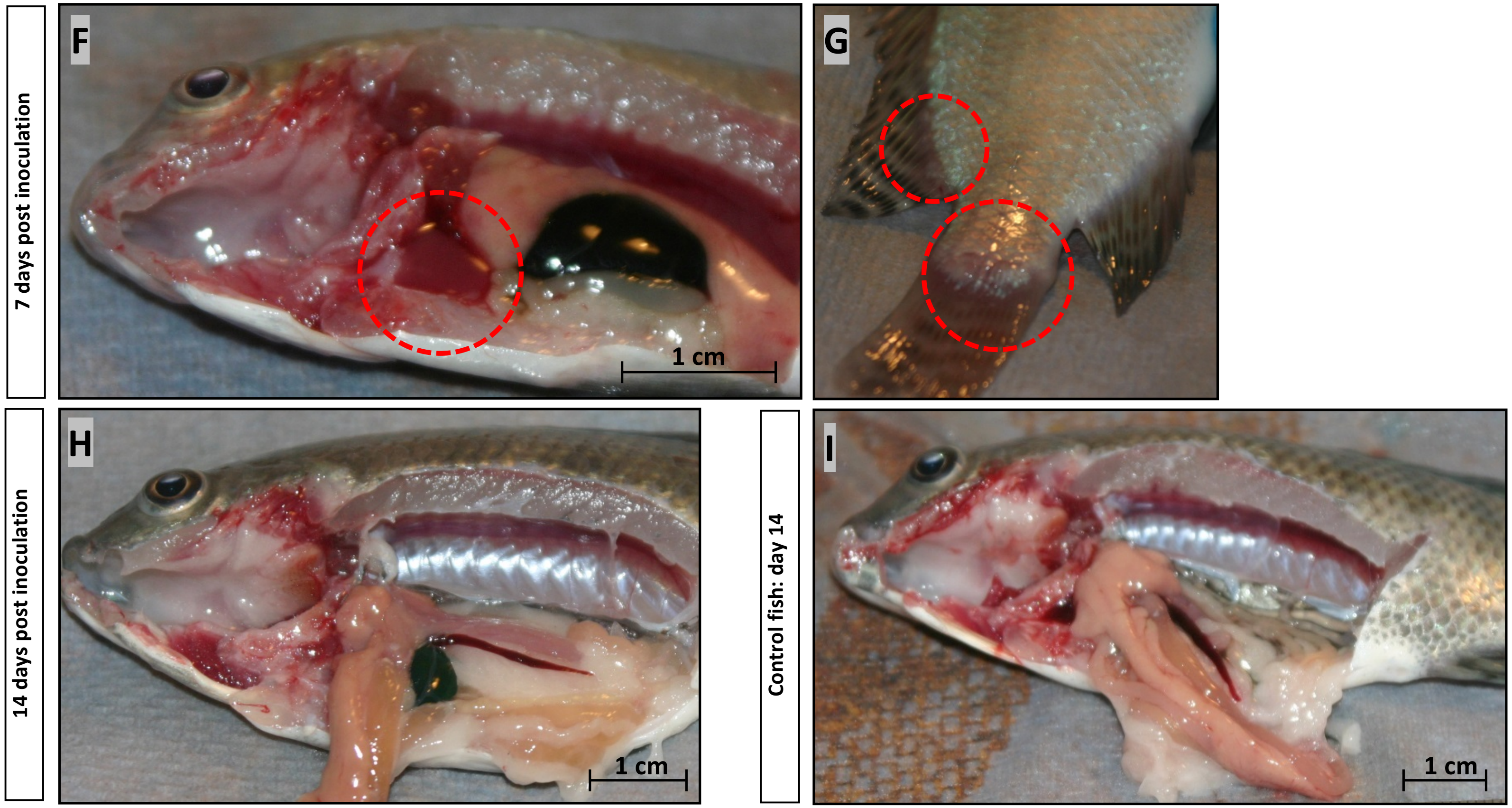

Figure 6.4 continued Clinical signs of disease and other observation from fish experimentally infected with *Streptococcus agalactiae*. [F] Fish sampled 7 days post inoculation with an enlarged heart [G] Fish sampled 7 days post inoculation with several lesion at the base of the dorsal and caudal fins [H] Fish sampled 14 days post inoculation with a relatively normal sized spleen and abdominal fat [I] Control fish that did not receive any bacteria and sampled on day 14.

During the sequential *S. agalactiae/S. iniae* combined challenge there were no mortalities or noticeable morbidity in any of the tanks. Yet there was a variety of clinical signs observed from 6 hours to 14 days post inoculation (Table 6.4). These included clinical signs that were seen in *S. agalactiae* and *S. iniae* challenges such as haemorrhagic intestinal content and lesions around fins (Figure 6.5). However, there were very few histopathology changes found. The brain of fish sampled 6 hours and 3 days after inoculation had EGC with thickening of the pia-mater seen in day 1 samples. At 6 hours after inoculation the spleen was shown to have accumulations of basophilic cells, after 1 day, vacuolation was observed and on day 3 and the spleen contained lots of blood cells.

For all three bacterial challenges (*S. agalactiae, S. iniae* and *S. agalactiae/S. iniae* combined) nephrocalcinosis was seen in kidney samples and parasites were found in the gills. However, these findings were also seen in the control fish (Figure 6.6). In addition to the bacterially challenged fish, control fish also has lesions on the jaw, which were assumed to be behavioural, and on caudal fins (Figure 6.5). Abdominal fat was also seen in the control fish (Figure 6.4 and 6.5), which is commonly seen in the Tropical Aquarium facilities when fish receive a frequent feeding regime using Skretting trout pellets. There were no other apparent pathological changes detected.

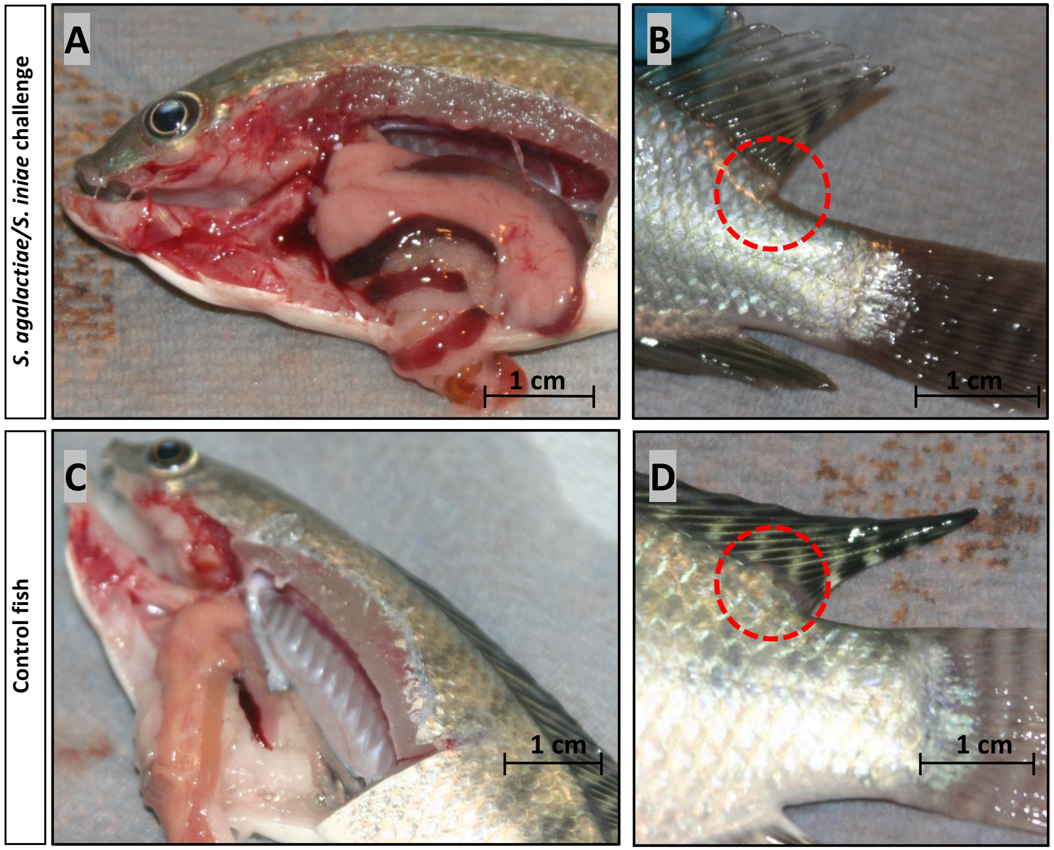

Figure 6.5 Clinical signs of disease and other observation from fish experimentally infected with *Streptococcus agalactiae* and *Streptococcus iniae* combined. [A] Fish sampled 6 hours post inoculation with bloody ascites [B] Fish sampled 3 days post inoculation with lesion at the base of the dorsal fin [C and D] Control fish that did not receive any bacteria and sampled on day 14. Fish present with a relatively normal sized spleen, abdominal fat but also a lesion at the base of the dorsal fin.

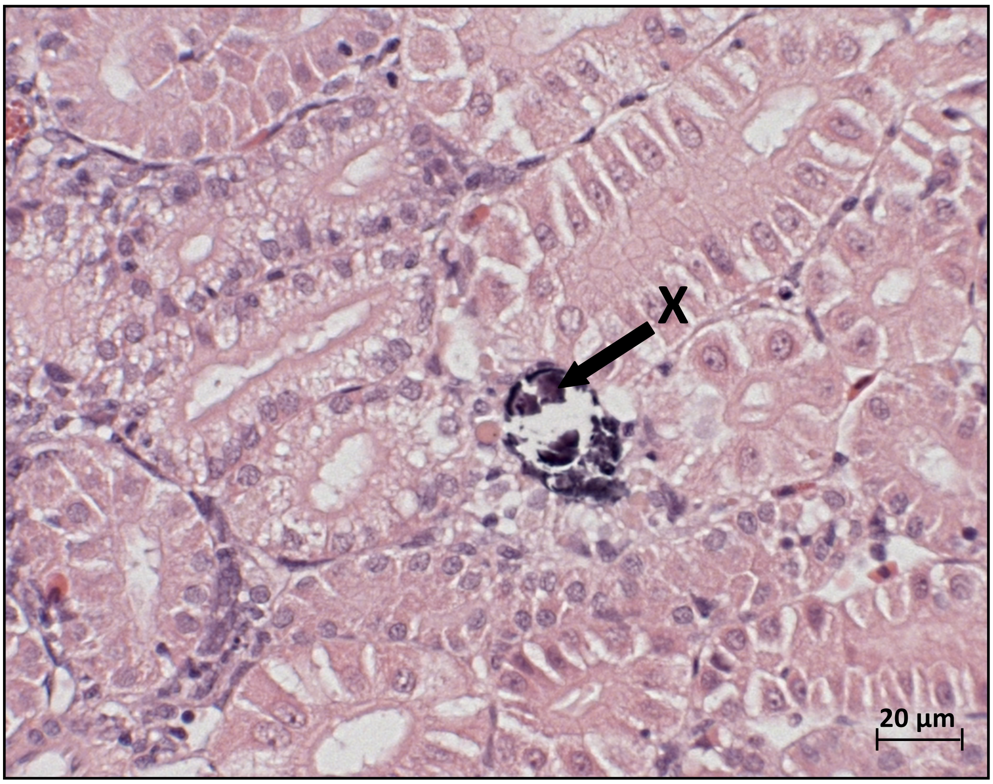

Figure 6.6 Evidence of nephrocalcinosis (X) in the kidney of a control tilapia (H&E). This fish was not exposed to any bacterial challenge.

6.4 Discussion

When the fish were inoculated with *S. agalactiae* and *S. iniae* combined four possible outcomes were hypothesised:

1. The fish would be overwhelmed and die very quickly: there would be higher mortality levels compared to the individual bacterial challenges.
2. There would be no impact: the mortality rate would be the same as individual bacterial challenges.
3. There would be a cumulative effect: there would be a higher total mortality rate compared to the individual bacterial challenges but there would be two 'waves' of mortality/morbidity caused by *S. agalactiae* and *S. iniae* individually.
4. There would be competition between the bacteria: there would be a lower mortality rate compared to the individual bacterial challenges.

Unfortunately, during the sequential challenges there were extremely low mortality rates (if any), which did not add any significant new information and failed to support any of the hypotheses. This was also found when the histopathology samples were examined. However, the sequential pathology study did suggest that signs of *S. agalactiae* infection occured very quickly after exposure. Although there was a low level of mortality there were still clear signs of disease progression indicating that the experimental design was suitable for investigating the development of clinical signs and histopathological changes during a time course experimental infection. Furthermore the vast majority of the clinical signs that were observed in the pilot *S. agalactiae* challenge, which had higher levels of mortalities, where also identified in the sequential study.

This was not the case for the *S. iniae* sequential challenge. Disease progression was not pronounced when the histopathology samples from different time points were examined.

This was probably a direct result of there being no mortalities or morbidities. An acute infection was not established, as it was in the pilot challenges (Chapter 5), and consequently there were also very few clinical signs of disease noticed. These findings were similar in the combined *S. agalactiae* and *S. iniae* challenge. It is difficult to ascertain whether this was a result of bacterial competition or a result of a dilution effect caused by there being two bacterial suspensions in one inoculation.

Following the successful reproduction of disease in the pilot challenges (Chapter 5) the lack of mortalities and morbidity in the first *S. iniae* challenge was surprising. Consequently, two bacterial challenges (one *S. agalactiae* and one *S. iniae*) were conducted simultaneously to some of the sequential challenges with the only variable being the fish batch. Since all other parameters such as water temperature, bacterial concentration etc. were exactly the same between these challenge groups it was shown that fish batch variation had an enormous effect on mortality rates. In fish batch A nephrocalcinosis was seen in samples of the kidney and parasites were found in the gills. Although parasites were also found in fish batch B, nephrocalcinosis was not apparent in either fish batch B or in pilot challenge. Nephrocalcinosis is the granular deposition of calcium or magnesium salts within the renal tubules and ducts of the kidney (Bruno, 1996; Lall, 2010). This kidney disorder has been linked to several dietary and environmental factors such as prolonged exposure to high levels of carbon dioxide (Bruno, 1996; Lall, 2010). The cause of nephrocalcinosis in fish batch A has not been determined however, it is not believed to be nutritional as all batches of fish received the same feed. Consequently, environmental factors caused by varied husbandry conditions may be the cause. Although fish originated from the same facility there was slight variation in their husbandry such as: frequency of grading, stocking density, feeding rate and the system they were grown in. These varied husbandry conditions between fish batches may have had a significant effect on the fish, this includes disease susceptibility. The actual cause of nephrocalcinosis and the large discrepancies between mortality rates is undetermined and was beyound of the scope of this study to investigate.

This study aimed to use molecular techniques, such as PCR, to assess *in vivo* bacterial distribution. However, bacterial identification in internal organs could not be achieved for *S. iniae*. As seen previously through Chen *et al*. (2007) and histopathology analysis from chapter 5, even in acute *S. iniae* infection the bacterial load in infected organs is low especially when compared with *S. agalactiae* infected tissue. In this study no such acute *S. iniae* infection was established therefore if bacteria were present in organs, their numbers were simply too low to be detected through PCR. An alternative strategy to study *in vivo* bacterial distribution would be the use of fluorescent proteins to act as an endogenous fluorescent tags. Such a methodology has been successfully tried by several researchers whereby a green or red fluorescent protein (GFP/RFP) was used to render bacteria visible and their invasion pathway traced in *in vivo* fish models (Chu and Lu, 2008; Ling *et al*., 2000; Singer *et al*., 2010). An *in vitro* study also demonstrated the potential of multi-coloured tagging system using fluorescent proteins (Andersen *et al*., 2006). Consequently, there is potential for transforming *S. agalactiae* and *S. iniae* with different coloured fluorescent protein for investigating a simultaneous infection in tilapia.

Due to the unforeseen effect fish batch variation had on mortality and morbidity levels, the investigation into a combined *S. agalactiae/S. iniae* challenge was limited. However, the overall experimental design appears to be appropriate. Disease progression was noticeable in the *S. agalactiae* sequential challenge and signs of infection were detectable as soon as 6 hours post inoculation. The main drawback in the study was the failure to identify *S. agalactiae* and *S. iniae* in infected tissue. An alternative method needs to be developed before further research in simultaneous challenges is performed.

6.5 References

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Chapter 7

General discussion

The aetiological agents of streptococcosis in fish were identified many decades ago yet aquaculture sectors throughout the world are still to this day suffering from this disease. Both *Streptococcus agalactiae* and *S. iniae* have the capacity to infect a wide range of fish hosts some of which supply a large proportion of the global fish farming market such as tilapia. China, in particular, has suffered from severe and extensive outbreaks of streptococcosis in cultured tilapia continuously for many years (Zhang *et al*., 2013). Such large-scale outbreaks in China resulted in a loss of approximately US$0.4 billion in 2011 (Chen *et al*., 2012).

As shown in Figure 7.1 when a comparison is made between countries that produced tilapia (FAO, 2013) and the literature on *S. agalactiae* and *S. iniae* outbreaks in tilapia there are some disparities. Overall, the countries which produce tilapia have mostly also reported streptococcosis outbreaks with the exception of those in Africa (Figure 7.1). Tilapia has been cultured in 23 out of 32 African countries with production from these countries accounting for 12.8% of the global tilapia production in 2002 (El-Sayed, 2006); yet to date there are no scientific literature reports of streptococcosis outbreaks in these countries. There are accounts and descriptions of streptococcosis outbreaks in Africa, such as those by Huchzermeyer and Henton (2011), however, there are no published scientific investigations. Research by Thrush *et al*. (2012) also found a regional bias in the reporting of emerging or new diseases whereby generally there are no reports from continental regions such as Africa even with their significant aquaculture production. It is unclear whether this is due to insufficient surveillance or under-reporting (Thrush *et al*., 2012). Either way this means there is no comprehensive record of global occurrences of streptococcosis.

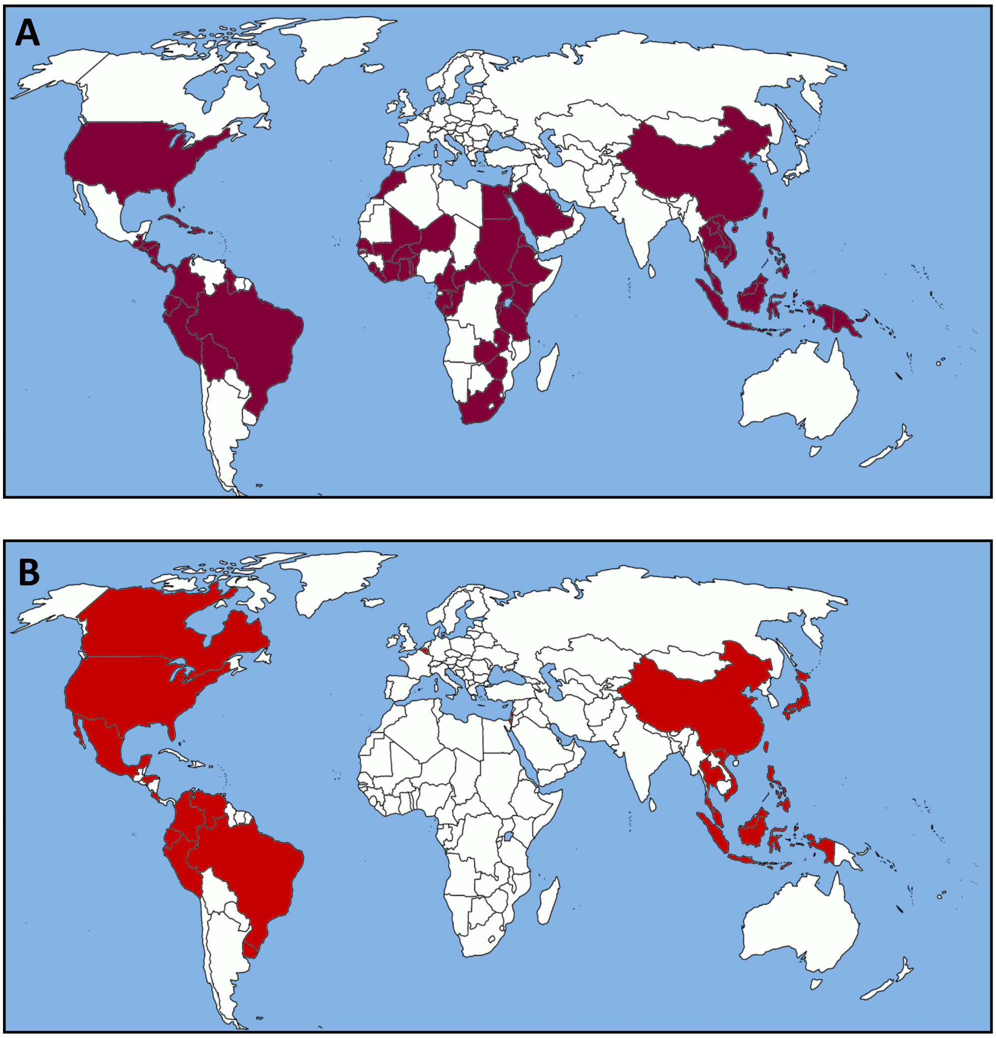

Figure 7.1 [A] Countries that produced tilapia from 2002 - 2012 (*Oreochromis* spp.) (FAO, 2013; FAO Fishery Statistics, 2013). [B] Countries that have reported *Streptococcus agalactiae* or *Streptococcus iniae* infection in tilapia from 1993 - 2013 (Table 1.2 and 1.3). NB: Nile tilapia is produce in other countries than highlighted on the map yet there is no record of their annual production through FAO.

The occurrence of streptococcosis outbreaks may yet become more severe through the impact of global warming. The repercussions of climate change are numerous, however, increasing water temperature is considered to have profound effects on infectious diseases of aquatic animals (Karvonen *et al*., 2010; Marcogliese, 2008). Climate change is thought to directly affect pathogen distribution but may also change host range and susceptibility as well as pathogen abundance (Marcogliese, 2008). It has already been shown that *S. iniae* can be transmitted from wild fish to cultured fish (Colorni *et al*., 2002; Zlotkin *et al*., 1998) and both *S. agalactiae* and *S. iniae* can infect a wide variety of fish species (Table 1.2 and 1.3).

Consequently, climate change has the potential to exacerbate streptococcosis outbreaks as fish ranges extend and there is the possibility of transmitting pathogens to new areas. Disease dynamics may also be altered; yet temperature-driven increase in disease occurrence may only occur for some certain pathogens (Karvonen *et al.*, 2010). *Streptococcus iniae* is more predominant in cooler water temperatures (15 °C to 24 °C) whereas *S. agalactiae* in higher water temperatures (24 °C to 26 °C) (Conroy, 2009; Salvador *et al*., 2005). Therefore as summarised by Karvonen *et al.* (2010): 'whilst there are pathogens that may benefit from climate warming, the prevalence of others may decrease or remain unaffected'. It remains uncertain what effects global warming will have on streptococcosis outbreaks, however, prevalence is already high and control of this disease remains important.

It is highly likely that the sustained presence of streptococcosis within tilapia farming is multifactorial. Prevention through vaccination is limited, as commercially available vaccines are not licensed for use in all countries producing tilapia. Furthermore, the available vaccines do not appear to be very effective. Although there has been a considerable amount of research conducted on vaccine development the heterogeneous nature of both *S. agalactiae* and *S. iniae* has limited their effectiveness at the field level. Pridgeon and Klesius (2013) believe that developing a polyvalent *S. agalactiae* vaccine that will protect against all *S. agalactiae* strains will be very difficult, 'if not impossible'. The application of a successful vaccination programme for *S. iniae* has also proved difficult as under vaccination pressure variations in capsular and polysaccharide structures have developed 'that enable the variant to evade vaccine-induced host immunity' (Zhang *et al*., 2014). Furthermore, reports have claimed that there is a trend to find a *S. iniae* outbreak followed by another outbreak caused by *S. agalactiae*, and vice versa (Conroy, 2009). Therefore a dual vaccination, to protect against both *S. agalactiae* and *S. iniae,* should ideally be investigated but testing of these would need a novel experimental model that incorporated simultaneous or co-infections. To produce a reliable and reproducible bacterial challenge model, irrespective of whether this is single or combined pathogens, recovery and identification of the pathogens is required. One of the constraints affecting streptococcosis reporting from natural infections in farmed fish species is the lack of clear phenotypic tables to distinguish bacterial groups from one another (Abbott *et al.*, 2003). As a result, there is no standardised identification protocol for these bacterial species making comparative microbial identification studies problematic. From the published literature, there does appear to be a reliance on the use of miniaturised identification systems such as the API 20 STREP system and there is a tendency for bacterial strains to be identified solely on their similarity to the species type strain. This study has highlighted there is a high degree of variability in the phenotypic characteristics of *S. agalactiae* and *S. iniae* strains and therefore more caution needs to be taken when using such identification techniques to confirm species identification.

The results presented in this study from the bacterial identification work performed pre and post-passage through fish, further highlighted the variability of API 20 STREP results. Consequently, the storage and sub-culturing of bacteria recovered from infected fish could have profound effects on its biochemical characteristics. This is a novel finding in relation to API 20 STREP results and one that has not been previously mentioned in the fish-pathogen literature for streptococcal species. This may explain some of the variation seen between research studies based on API 20 STREP profiles. Additionally, since the API 20 STREP system was developed for mammalian strains researchers in fish diseases have deviated from the manufacturer's instructions, for example lower incubation temperatures, making comparison between results problematic. As a result of the work performed in this study, an alternative method for analysing results from an API 20 STREP was proposed. Rather than the 20 reactions being coded into a numeric profile, users could improve reliability by only focusing on particular individual assay results, which give consistent results. These include consistent negative and positive responses.

A robust and reliable method was developed during this study for the identification of *S. agalactiae* and *S. iniae* strains. This incorporated standard primary identification tests, serological and biochemical assays and molecular tests. Such an identification protocol is fundamental when working with two closely related bacterial species. Additionally, if such a protocol was universally implemented for the identification of *S. agalactiae* and *S. iniae* this would make comparisons between research groups possible and help highlight intra-species strain variations in fish isolates. Understanding strain variability is a key step in vaccine development. Expanding research to incorporate serotyping and genotyping would further identify intra-species relationships and provide valuable information on their epidemiology.

However, not all facilities will have the resources, time or funding to conduct extensive diagnostics tests. Nevertheless, the identification protocol recommended in this study uses a minimal amount of tests to confirm the identification of these two pathogens. Furthermore, the approach suggested for conducting identification tests on suspected *S. agalactiae* and *S. iniae* cases would improve confidence and reliability in the results obtained.

The clinical signs presented in fish infected with *S. agalactiae* or *S. iniae* are generally indistinguishable, however, from the *in vitro* and *in vivo* work performed in this study, it was established that the pathogenesis of *S. agalactiae* and *S. iniae* are actually quite different. *In vitro* assays can reduce the number of 'protected' animals used in scientific research and certain assays were able to reproduce key stages in disease progression. Animal experimental work, including the use of fish, must comply with the UK Home Office requirement for reduction, refinement and replacement. To do this the *in vitro* screening of virulent and avirulent strains would be a valuable method prior to experiments in fish, reducing the use of animals in research. However, the results of this study also demonstrated the limitations of some *in vitro* assays reinforcing the necessity for *in vivo* studies. The mechanisms employed by pathogens to cause disease must still be identified; this is usually achieved by investigating virulence factors.

The study of virulence factors has increased over the last decade as it not only contributes to the understanding of bacterial pathogenesis but virulence factors can also serve as novel targets in vaccine development. However, the mere presence of a virulence gene does not always equate to activation and expression of the virulence factor. Therefore, a standard methodology for assessing virulence factors is *in vivo* screening of transposon mutants for loss of virulence (Buchanan *et al*., 2005). This technique was not possible during this study due to regulations surrounding the creation of genetically modified organisms. Additionally, caution must be taken to avoid mistaking factors that affect bacterial growth for factors that contribute to bacterial virulence. As stated by Allen *et al*. (2014) virulence factors are ‘components that are non-essential to *in vitro* growth’. Only a few molecular experiments were conducted in this study yet the *in vitro* assays that were developed assessed the expression of certain virulence factors. As such, results indicated that virulent *S. agalactiae* strains would be more likely to spread systemically within the host whereas *S. iniae* strains would be more localised. Indeed *in vivo* investigations from other researchers and findings from this study support this theory (Chen *et al*., 2007). Histopathology findings clearly demonstrated a systemic spread of *S. agalactiae* with high bacterial loads in all the organs examined. Whereas, *S. iniae* was observed in considerably fewer organs of infected fish and bacterial numbers were substantially lower. Consequently there could be a threat of prolonged infection on farms if two bacterial species are present concurrently and they employ different mechanisms of pathogenesis.

Given the lack of research into concurrent infections it was unclear what would happen when two bacterial species were combined in the infection model. *In vitro* experiments indicated that there would be either no interaction between bacterial species or they would coexist. *In vivo* challenges in wax moth larvae (*Galleria mellonella*) contradicted this conclusion, suggesting pathogens interacted with one another in a competitive manner resulting in lower than expected mortalities. Although experiments such as these can be informative and provide valuable information on bacterial interactions it is the underlying bacterial-host interactions that are principally responsible for disease outcomes. It was therefore essential to assess the outcome of a concurrent infection in tilapia. This required a robust and reproducible infection model for both *S. agalactiae* and *S. iniae* simultaneously in a single host.

There are various exposure routes that have been used to induce *Streptococcus* infections in fish such as gill and nares inoculation, bath immersion and cohabitation (Evans *et al*., 2000; McNulty *et al*., 2003b; Mian *et al*., 2009; Rodkhum *et al*., 2011; Shoemaker *et al*., 2000). Such methods are considered to provide a more natural mode of infection and possibly a more natural response (McNulty *et al*., 2003). Consequently, these methods are considered to be more representative of actual transmission and infection in farming environments (Mian *et al*., 2009). An intraperitoneal (i.p.) injection, however, has the drawback that portions of the natural defense barriers of the host, and thus immune response, are by-passed (Jiménez *et al*., 2011). With an i.p. inoculation the exact amount of bacterial entering the fish is known and higher infection rates can often be achieved compared with the more natural routes of bacterial exposure. As a result, there is generally reduced variability with i.p. inoculation, which is why this remains the most common challenge method used in aquaculture disease research. The scope of this study was to begin a preliminary investigation into concurrent bacterial infections therefore i.p. inoculation appeared appropriate for purpose. However, since bacteria were administered artificially through i.p. injection further research should investigate different challenge routes such as bath immersion as used in other simultaneous studies (Crumlish *et al*., 2010).

In this study administering *S. agalactiae* and *S. iniae* through i.p. injection appeared to provide a reliable and reproducible experimental infection model which produced characteristic clinical signs in the fish. Unfortunately, for the subsequent *S. agalactiae and S. iniae* challenges in Chapter 6, fish batch variation appeared to be a very large uncontrolled variable. This was not anticipated particularly given the reproducibility of the infection model conducted previously during the pilot testing. Nevertheless, other researchers have investigated variation of susceptibility of fish to bacterial infection. In addition to a genetic basis of natural resistance (Langevin *et al*., 2012), aspects such as exposure to stress (Ndong *et al*., 2007) and inappropriate nutrition (Plumb and Hanson, 2011) have been shown to influence disease susceptibility under experimental conditions. Stress, induced by factors such as handling, stocking density and suboptimum environmental parameters can result in reduced immune capabilities within the fish and subsequently trigger disease outbreaks. However, research by Costas *et al*. (2011) has shown that repeat acute stress can actually increase both disease resistance and innate immune mechanisms in handled Senegalese sole (*Solea senegalensis*). In the study presented here, it is not known what caused the variation in disease susceptibility; however, future work would require a more diligent control of all aspects of fish husbandry in an attempt to minimise fish batch variation.

Even taking into account the issues raised during the fish challenge studies, it was clear that *S. agalactiae* and *S. iniae* employed different virulence mechanisms, and the co-infection model indicated that a simultaneous infection did not result in a cumulative effect on mortality. This suggests that *S. agalactiae* or *S. iniae* may either coexist or have no interaction. However, there is a concern that by using different inoculation concentrations between the individual and the concurrent challenges a diluent effect may have been produced. This could be rectified in future studies by varying the inoculum concentration. Furthermore, due to overwhelming abundance of *S. agalactiae* in infected tissues it is difficult to ascertain the location and quantities of *S. iniae* in organs. Subsequently, monitoring infectious pathways becomes challenging. Therefore, as highlighted in Chapter 6, using endogenous fluorescent tags through the application of fluorescent proteins would be beneficial. This process would clarify the pathogenesis of these two bacterial species and their interaction, if any, when they are within the same host at the same time.

The intention of this study was to gain insight into *S. agalactiae* and *S. iniae* infections and explore any interactions. There are several reasons why this is of importance. Both *S. agalactiae* and *S. iniae* can be misidentified on primary bacteriological identification, *S. agalactiae* and *S. iniae* infected fish show similar clinical signs and both pathogens can be found on the same farm during a disease outbreak. This complicates diagnosis, which is required to allow for a more specific treatment or preventative measures. Understanding how to identify and differentiate the two pathogens will allow the application of appropriate vaccination programmes, which is particularly important since vaccines target a specific species due to the lack of cross protection. Furthermore, understanding the natural history and pathogenesis of each organism may allow the development of separate control strategies. Confusion between the two pathogens may also render epidemiological studies invalid since data from two separate infections or even co-infections may be indistinguishable. It is highly improbable that useful associations will be found unless the data collected is clearly defined according to the pathogens present in the outbreak.

In addition to the confusion between the two pathogens there is evidence of simultaneous infections, and without an understanding of how the organisms interact within a host it is impossible to interpret clinical evidence of co-infections. Without such an understanding there are many questions a clinician must ask that could not be answered. For example: Is one pathogen doing all the damage? Is a simultaneous infection more or less serious than a single species infection? Are there any treatments which will combat both species? What if only one species was treated for?

The original objectives set out at the beginning of this study are restated below along with a brief summary of what was achieved:

(1) To evaluate and determine the most useful techniques for the detection and identification of these *S. agalactiae* and *S. iniae*.

It was determined that a *S. agalactiae* and *S. iniae* identification protocol should consist of [1] primary assays such as: Gram staining, motility, oxidase test [2] secondary assays such as Slidex Strepto-kit, starch hydrolysis and API 20 STREP test looking particularly at individual results and [3] tertiary assays which involve a duplex PCR.

(2) To identify the expression of virulence factors *in vitro* for *S. agalactiae* and *S. iniae* and compare any inter and intra-species variation.

Results demonstrated that the aquatic *S. agalactiae* and *S. iniae* strains tested possessed the same virulence factors including the possession of a capsule but their ability to break down blood, survive in blood and resist complement-mediated killing differed.

(3) To assess if there is competition or coexistence between *S. agalactiae* and *S. iniae in vitro*.

It was found that competition between *S. agalactiae* and *S. iniae in vitro* was inconsistent between different experimental systems. Results indicated that there was either no interaction between bacterial species or they coexisted during *in vitro* competition assays. Whereas, an *in vivo* model utilising wax moth larvae (*G. mellonella*) found that during a simultaneous infection with *S. agalactiae* and *S. iniae* the total levels of larvae mortality were lower than expected which indicated that the pathogens may be interacting with one another in a competitive manner.

(4) To develop a robust and reliable challenge model for *S. agalactiae* and *S. iniae* in Nile tilapia using intraperitoneal (i.p.) injection.

In this study it was apparent that fish infected with *S. iniae* experienced an acute infection with morbidity/mortality occurring 1 – 3 days after exposure. Whereas, in the *S. agalactiae* challenge fish showed a more chronic infection with mortalities occurring 1 – 6 days after exposure. Findings clearly demonstrated a more systemic spread of infection during a *S. agalactiae* challenge as there were high bacterial loads in all the organs examined. Whereas, *S. iniae* was observed in fewer organs of infected fish and bacterial numbers were substantially lower.

(5) To perform a sequential challenge for [1] *S. agalactiae* [2] *S. iniae* and [3] *S. agalactiae* and *S. iniae* in Nile tilapia.

Due to an unforeseen effect that fish batch variation had on the challenge model the results produced from the sequential challenge were limited.

Although these studies provided some insights into concurrent infections the complexities of such infections under natural and experimental conditions will necessitate considerably more research to provide a thorough understanding. This study provides a solid foundation upon which future work can expand on, particularly given the proposed differences in disease establishment of these two bacterial pathogens in fish.

7.1 Future work

Concurrent infections are complex in natural conditions as well in experimental studies and subsequently will require a substantial amount of research to provide sufficient data to fully understand the processes involved. This study provides a solid foundation upon which future work can expand on, particularly given the proposed differences in disease establishment of these two bacterial pathogens in fish. Areas highlighted for further investigation include: [1] investigating bacterial extracellular products in particular those that may regulate or restrict bacterial growth. This could be achieved through research into quorum sensing and identifying the expression of virulence factors; which have been shown to vary during different stages of growth (Unnikrishnan *et al*., 1999) and during interactions with other bacterial species (Bassler, 1999). Such research may prove beneficial for the design of novel vaccines. [2] Different inoculum concentrations and ratios of *S. agalactiae* to *S. iniae* used in the infection model could be investigated in addition to alternative exposure routes. This would reflect a more natural infection process and thus provide a more in-depth analysis of the impact of concurrent infections on fish farms. [3] Environmental conditions, such as water temperature, have been shown to influence the occurrence of diseases by either favouring the growth of the pathogen or causing the host to become stressed through suboptimal husbandry conditions. Thus, conducting concurrent infections within a range of different environment conditions may identify factors that favour the outbreak of disease caused by particular bacterial species. Finally [4] techniques such as quantitative polymerase chain reaction (qPCR) and Real-time PCR can be incorporated into the challenge design. These tools can identify the presence and quantity of pathogens in different organs and additionally analyse the interrelationship between the virulence of a bacterium and the adaptive immune response of a fish (Dixon and Becker, 2012). These research areas would provide much needed information regarding *S. agalactiae* and *S. iniae* infections and contribute to the long-term objective of improving the management and control of streptococcosis.

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Appendix

Staining protocols

1. Gram stain for bacterial cultures

Reagents:

Crystal violet solution (2%)

Crystal violet	2 g
95% ethanol	20 ml
Ammonium oxalate	0.5 g
Distilled water	80 ml

Dissolve the crystal violet in the ethanol solution. Dissolve ammonium oxalate in distilled water. Mix these two solutions, allow to stand for 24 hours then filter.

Gram's iodine

Iodine	1 g
Potassium iodide	2 g
Distilled water	300 ml

Dissolve the potassium iodide in 5 ml of distilled water, add the iodine and allow to dissolve. Add the remaining water. All to stand for 24 hours then filter.

Safranine solution

Safranine	0.25 g
95% ethanol	10 ml
Distilled water	90 ml

Dissolve the safranine in ethanol and add the distilled water. Allow to stand for 24 hours then filter.

Protocol:

Institute of aquaculture – Bacteriology lab method

Crystal violet	1 minute
Iodine	1 minute
Acetone	2-3 seconds
Safranin	2 minutes
Tap water	5 seconds

Result:

Gram positive bacteria	Pink/red
Gram negative bacteria	Blue/purple

2. Giemsa staining for blood smears

Reagents:

Working Giemsa solution

Giemsa	0.5 ml
Distilled water	10 ml

70% methanol

Methanol	70 ml
Distilled water	30 ml

Protocol:

Institute of Aquaculture – Histopathology lab method

70% methanol	3 minutes
Giesma stain	10 minutes
Distilled water	5 seconds

Result:

Nuclei	Red to violet
Lymphocytes	Plasma blue
Monocytes	Plasma dove-blue
Neutrophilic granulocytes	Granules light violet
Eosinophilic granulocytes	Granules red to grey-blue
Basophilic granulocytes	Granules dark-violet
Thrombocytes	Violet
Erythrocytes	Red

3. Rapid Romanowsky stain

Reagents:

<u>70% methanol</u>

Methanol	70ml
Distilled water	30 ml

Protocol:

Institute of Aquaculture – Histopathology lab method

70% methanol	3 minutes
Rapid Romanowsky stain B	30 seconds
Rapid Romanowsky stain C	30 seconds
Distilled water	5 seconds

Result:

Nuclei	Red to violet
Lymphocytes	Plasma blue
Monocytes	Plasma dove-blue
Neutrophilic granulocytes	Granules light violet
Eosinophilic granulocytes	Granules red to grey-blue
Basophilic granulocytes	Granules dark-violet
Thrombocytes	Violet
Erythrocytes	Red

4. Anthony's capsule stain

Reagents:

Crystal violet solution (1%)

Crystal violet	2 g
95% ethanol	20 ml
Ammonium oxalate	0.5 g
Distilled water	80 ml

Dissolve the crystal violet in the ethanol solution. Dissolve ammonium oxalate in distilled water. Mix these two solutions, allow to stand for 24 hours then filter.

Copper sulphate (20%)

Copper sulphate	20 g
Distilled water	100 ml

Milk broth

Skimmed milk powder	4.75 g
Distilled water	500 ml

Autoclave for 20 minutes at 121°C.

Protocol:

Based on that from Hughes and Smith (2007).

1% crystal violet	2 minutes
20% copper sulphate solution	Gently rinse

A cover slip is then added to the slide using Pertex.

Result:

Bacteria with capsule	Bacterial cells and the proteinaceous background will appear purple with a the surrounding transparent halo

5. Gram staining of tissue sections

Reagents:

<u>1% aqueous neutral red</u>

Neutral red	1 g
Distilled water	100 ml

Dissolve the neutral red in distilled water and filter before use.

<u>Gram's iodine</u>

Iodine	1 g
Potassium iodide	2 g
Distilled water	300 ml

Dissolve the potassium iodide in 5 ml of distilled water, add the iodine and allow to dissolve. Add the remaining water. All to stand for 24 hours then filter.

<u>Crystal violet solution</u>

Crystal violet	2 g
95% ethanol	20 ml
Ammonium oxalate	0.5 g
Distilled water	80 ml

Dissolve the crystal violet in the ethanol solution. Dissolve ammonium oxalate in distilled water. Mix these two solutions, allow to stand for 24 hours then filter.

Protocol:

Institute of Aquaculture – Histopathology lab method

Xylene (Dewax I)	3 minutes
Xylene (Dewax II)	2 minutes
Absolute alcohol I	2 minutes
Methylated spirit	1 minute
Tap water	45 seconds
Crystal violet solution	2 minutes 30 seconds
Gram's iodine wash	Wash slide until metallic precipitate is washed away
Gram's iodine	2 minutes 30 seconds
Tap water	Wash slide to remove Gram's iodine
Acetone	Wash slide until section appears colourless
Tap water	45 seconds
1% neutral red	1 minute
Tap water	45 seconds
Absolute alcohol II	2 minutes
Absolute alcohol III	1 minute 30 seconds
Xylene (Clearing)	5 minutes
Xylene (Coverslip)	As required

A cover slip is then added to the slide using Pertex.

Result:

Gram positive bacteria	Blue/purple
Gram negative bacterial	Pink/red
Nuclei	Red

6. Haematoxylin and eosin staining

Reagents:

Mayer's haematoxylin

Haematoxylin	2 g
Sodium iodate	0.4 g
Potassium alum	100 g
Citric acid	2 g
Chloral hydrate	100 g
Distilled water	2000 ml

Allow the haematoxylin, potassium alum and sodium iodate to dissolve overnight in the distilled water. Add chloral hydrate and citric acid, then heat until boiling. Continue boiling for 5 minutes.

1% acid alcohol

Methylated spirits	1980 ml
Hydrochloric acid	20 ml

Scott's tap water substitute

Sodium bicarbonate	3.5 g
Magnesium sulphate	20 g
Tap water	1000 ml

Dissolve by heating. Add a few thymol crystals to act as a preservative.

Eosin solution

Putt's eosin	80 ml
1% eosin	240 ml

Putt's eosin

Eosin yellowish	4 g
Potassium dichromate	2 g
Picric acid	40 ml
Absolute ethanol	40 ml
Distilled water	320 ml

Dissolve the eosin and potassium dichromate in the ethanol. Add the distilled water followed by the picric acid.

1% eosin

Eosin yellowish	20 g
Distilled water	2000 ml

Dissolve the eosin yellowish in 500 ml of distilled water then top up with distilled water for a total volume of 2000 ml.

Protocol:

Institute of aquaculture – Histopathology lab method

Xylene (Dewax I)	3 minutes
Xylene (Dewax II)	2 minutes
Absolute alcohol I	2 minutes
Methylated spirit	1 minute 30 seconds
Tap water	45 seconds
Haematoxylin ‘z’	5 minutes
Tap water	45 seconds
1% acid alcohol	3 quick dips
Tap water	1 minute
Scott’s Tap Water Substitute	30 seconds
Tap water	45 seconds
Eosin	5 minutes
Tap water	45 seconds
Methylated spirit	30 seconds
Absolute alcohol II	2 minutes
Absolute alcohol III	1 minute 30 seconds
Xylene (Clearing)	5 minutes
Xylene (Coverslip)	As required

A cover slip is then added to the slide using Pertex.

Result:

Nuclei	Blue/Black
Cytoplasm and muscle fibres	Deep pink
Collagen	Light pink
Red blood cells and eosinophil	Bright orange/red

Media and solution preparation

1. STE and TE buffer

Tris 1M

Tris	1.21 g
MilliQ water	10 ml

EDTA 0.5M

As mentioned previously

NaCl 5M

NaCl	2.93 g
MilliQ water	10 ml

Working STE buffer

Tris 1M	1 ml
EDTA 0.5M	0.2 ml
NaCl 5M	2 ml
MilliQ water	96.8 ml

Working TE buffer

Tris 1M	1 ml
EDTA 0.5M	0.2 ml
MilliQ water	98.8 ml

2. 0.5 x Tris-acetate-EDTA (TAE) buffer

0.5M Ethylenediamine tetraacetic acid (EDTA)

EDTA disodium salt	93.05 g
Distilled water	500 ml

Add the EDTA disodium salt into 400 ml distilled water and adjust the pH to 8.0 using 1M NaOH. Top up the solution to a final volume of 500ml.

50 x TAE buffer

Tris base	242 g
Distilled water	1000 ml
Glacial acid	57.1 ml
0.5M EDTA	100 ml

Dissolve the Tris base in approximately 750 ml of deionized water. Add the glacial acid and EDTA and adjust the solution to a final volume of 1000ml with deionised water.

Working solution of TAE is made by dilution 5 ml of 50 x TAE buffer in 500 ml of distilled water.

3. Lysis buffer

Citric acid 0.1M

1% Tween 20

4. Phosphate buffered saline (PBS)

$NaH_2PO_4 2H_2O$	0.438 g
$Na_2HPO_4 2H_2O$	1.280 g
NaCl	4.385 g
Distilled water	500 ml

pH adjusted to 7.2 using a 1M HCl. Autoclave for 20 minutes at 121°C.

5. 2 x Sample buffer

0.5M Tris-HCl pH 6.8	2.5 ml
Glycerol	2.0 ml
10% SDS	4.0 ml
Dithiothreitol (DTT)	0.31 g
Bromophenol blue	2.0 mg
Distilled water	10 ml

6. SDS-PAGE acrylamide gel

NEXT GEL® 12.5% solution	12.5 ml
TEMED	7.5 µl
Ammonium persulfate	75 µl

7. Coomassie Brilliant Blue R-250 solution

Coomassie Brilliant Blue R-250 solution	0.25% w/v
Methanol	40%
Acetic acid	10%
Distilled water	50%

8. De-staining solution

Methanol	40% (v/v)
Acetic acid	10% (v/v)

9. SSTNE extraction buffer

NaCl	17.5 g
Tris Base	6.05 g
EGTA	76 mg
Spermidine	72 mg
Spermine	52 mg

EDTA 0.2M	1 ml
Distilled water	1 litre

After adding all the ingredients autoclave buffer at 121°C for 15 minutes.