GHENT UNIVERSITY

FACULTY OF VETERINARY MEDICINE
accredited by EAEVE

TOWARDS SUSTAINABLE DOVER SOLE (*SOLEA SOLEA*) LARVICULTURE

Contributing to the exploration of factors influencing its success

Evelien De Swaef

Evelien De Swaef (2019)

TOWARDS SUSTAINABLE DOVER SOLE (*SOLEA SOLEA*) LARVICULTURE

Contributing to the exploration of factors influencing its success

Cover design: Evelien De Swaef

Cover Photo Front: *Solea solea* larva, Evelien De Swaef

Cover Photo Back: *Solea solea* adult, Yves Adams, Vilda photography, www.vildaphoto.net

Printed by University Press, Wachtebeke, Belgium. www.universitypress.be

GHENT UNIVERSITY

FACULTY OF VETERINARY MEDICINE
accredited by EAEVE

TOWARDS SUSTAINABLE DOVER SOLE (*SOLEA SOLEA*) LARVICULTURE

Contributing to the exploration of factors influencing its success

Evelien M. E. De Swaef

Dissertation submitted in fulfillment of the requirements for the degree of Doctor in Veterinary Science (PhD)

Promotors:

Prof. dr. A. Decostere

Prof. dr. W. Van Den Broeck

Department of Morphology

Faculty of Veterinary Medicine

Ghent University

2019

Promoters

Prof. dr. Annemie Decostere

Faculty of Veterinary Medicine, UGent

Prof. dr. Wim Van Den Broeck

Faculty of Veterinary Medicine, UGent

Members of the Examination Committee

Prof. dr. Siska Croubels

Chair of the Examination Committee

Faculty of Veterinary Medicine, UGent

Prof. dr. Katleen Hermans

Secretary of the Examination Committee

Faculty of Veterinary Medicine, UGent

Prof. dr. Peter Bossier

Faculty of Bioscience Engineering, UGent

Prof. dr. Koen Chiers

Faculty of Veterinary Medicine, UGent

Dr. Marie-Laure Bégout

Unité Halieutique Gascogne Sud, Ifrémer, France

Dr. Anamaria Rekecki

IMAQUA BVBA, Melle

Mr. Marco Dubbeldam

Stichting Zeeschelp, the Netherlands

Table of contents

List of abbreviations

AASW	autoclaved artificial seawater
ARA	arachidonic acid
ASW	artificial seawater
BHIB	brain heart infusion broth
BU	biological unit
CFU	colony forming unit
CI	confidence interval
CT	multiplication of concentration and contact time
DAH	days after hatching
DAPI	4',6-diamidino-2-fenylindole
DGGE	denaturing gradient gel electrophoresis
DHA	docosahexaenoic acid
DPF	day post-fertilisation
DPI	day post-inoculation
EPA	eicosapentaenoic acid
EIA	enzyme immunoassays
ELS	early life stage
EU	experimental unit
FA	fatty acids
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration of the United States
FG	foregut
FOS	fructooligosaccharide
GOS	galactooligosaccharide
GRAS	generally recognized as safe
H_2O_2	hydrogen peroxide
HIER	heat-induced epitope retrieval
HPI	hypothalamic-pituitary-interrenal
IMARES	Institute for Marine Resources & Ecosystem Studies
LC_{50}	lethal concentration resulting in 50% mortality
MA	marine agar
MB	marine broth
MG	midgut
MHC	major histocompatibility complex
MOS	mannanoligosaccharide
MSY	maximum sustainable yield
MS 222	tricaine methanesulfonate
O_3	ozone
OECD	Organisation for Economic Co-operation and Development
Os_2O_4	osmium tetroxide
OU	observational unit
PAA	peracetic acid
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PCR-DGGE	polymerase chain reaction denaturing gradient gel electrophoresis
SEM	scanning electron microscopy
TCBS	thiosulfate citrate bile sucrose agar
TEM	transmission electron microscopy
TSB	tryptic soy broth
UPLC-MS/MS	ultra-performance liquid chromatography coupled to tandem mass spectrometry

Chapter 1 INTRODUCTION

The world population is expected to grow towards 9.6 billion people in 2050 (UN, 2014), creating a challenge to feed the population in a sustainable manner. In this respect, aquaculture is the fastest growing sector to produce animal protein in the world, accounting for approximately half of the fish consumed globally and representing at least 598 different aquatic species (Figure 1.1) (FAO, 2018). Furthermore, with the stagnation of capture fisheries production, an ever-increasing demand for fish through aquaculture production is to be expected. Further expansion of the aquaculture sector requires not only an intensification but also a diversification of the cultured species.

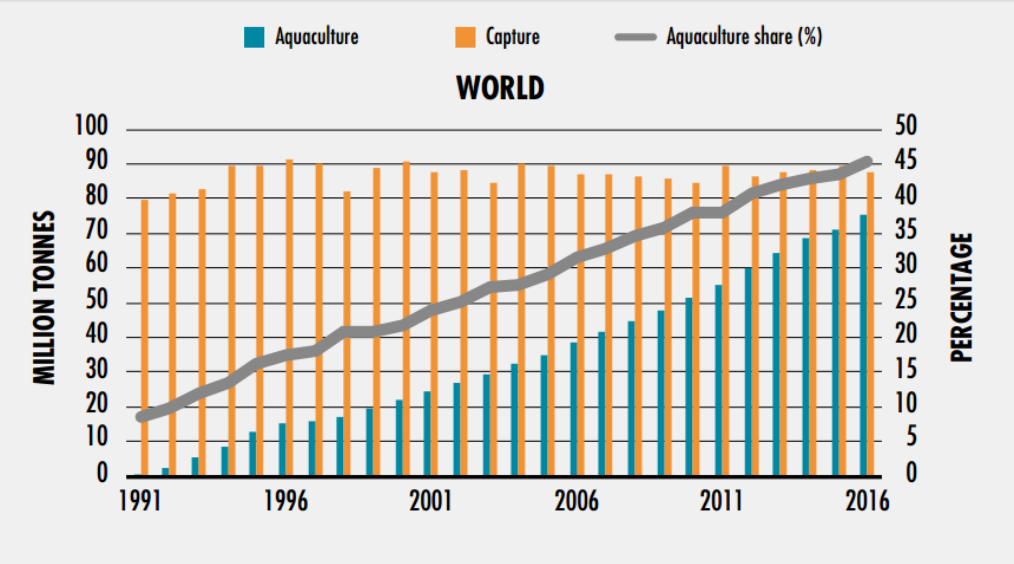


Figure 1.1 - Aquaculture contribution to total fish production (excluding aquatic plants) (FAO, 2018).

In 2016, world's food aquaculture production exceeded 80 000 000 tonnes and consisted of 68% finfish, 21% mollusks, 10% crustaceans and 1% miscellaneous animal species. Globally, finfish aquaculture is dominated (88%) by freshwater species. However, compared with the low production quantity, diadromous and marine fish have a higher market value. Focusing on European aquaculture, inland production is limited to 502 000 tonnes of finfish, whereas marine and coastal aquaculture produce 2 443 000 tonnes, constituting of 75% finfish and 25% mollusks (FAO, 2018). European marine finfish production is dominated by three main species; Atlantic salmon (*Salmo salar*, 1 492 000 tonnes in 2015), Gilthead seabream (*Sparus aurata*, 134 000 tonnes in 2015) and European seabass (*Dicentrarchus labrax*, 144 000 tonnes in 2015) (European Environment Agency, 2018).

European flatfish aquaculture mainly focusses on turbot (*Scopthalmus maximus*) and Atlantic halibut (*Hippoglossus hippoglossus*). For both species, yearly production fluctuates around 75 000 tonnes (FAO, 2018). Sole is a promising aquaculture candidate as the flesh is highly appreciated in culinary circles and has a high market value. Sole landings and aquaculture commonly refer to two species:

Dover sole (*Solea solea*) and Senegalese sole (*S. senegalensis*). Both species are closely related and almost indistinguishable to consumers. The limited number of market studies and reports on production costs combine and mutually compare both species (Imsland et al., 2003; Bjørndal et al., 2015; FAO, 2018). Dover sole is of major economic importance for the North Sea fisheries, representing 40% of the total landing value in Belgium and may therefore be considered one of the most relevant species in this area. Furthermore, due to indications of a limited market growth for species as European seabass and Gilthead seabream (Bjørndal et al., 2016; FAO, 2018), the aquaculture sector developed a renewed interest in Dover sole to diversify its operations. Indeed, sole was already recognized as a promising aquaculture species in the 1980s. However, technical and disease problems proved to be important drawbacks of the sole farming industry (Howell, 1997; Dinis et al., 1999). Large research efforts were undertaken, allowing to surpass several difficulties by improving breeding, husbandry and nutrition. However, sole aquaculture still faces important challenges such as high production costs due to feed prices and difficult supply of juveniles (Bjørndal & Guillen, 2014).

In what follows, an overview of Dover sole morphology, natural occurrence and aquaculture is given. Furthermore, the status and challenges in the larviculture of marine flatfish with special attention to Dover sole are discussed, hereby focusing on disease prevention as well as the research tools available.

1.1 DOVER SOLE - MORPHOLOGY

Dover sole is a benthic flatfish species that lives on sandy and muddy bottoms, with eyes and snout on the right hand side (Figure 1.2). The body is oval-shaped with a rounded head and the colour of the eyed side may vary between grey, brown and grey-brown with dark spots depending on the substrate. The pectoral fin has a clear black spot close to the margin. In contrast, the blind side is non-pigmented (FAO, 2018). Adults feed on polychaete worms, small soft-shelled bivalves, small fish and crustaceans. Dover sole can live for 40 years and reach a maximum length of 70 cm. Sexual maturity is reached at the age of 4 years (approximately 25 to 35 cm length), with males maturing slightly smaller and younger than females (Fishbase, 2018).

Figure 1.2 - Dover sole adult exhibits an oval-shaped body with rounded head. The eyed side is pigmented and the colour may vary between brown and grey. The pectoral fin has a distinctive black spot. The blind site is non-pigmented. (photo by Misjel Decleer)

1.2 DOVER SOLE - NATURAL OCCURRENCE

1.2.1 Distribution

Dover sole has a wide geographical distribution range, from the Trondheim fjord (Norway) till Senegal as well as the along the coasts of the Mediterranean sea (Figure 1.3) (Rijnsdorp et al., 1992; FAO, 2018). This species does not undertake large migrations but is considered to be relatively stationary (Rijnsdorp et al., 1992). Therefore, it is assumed that Dover sole in European waters belongs to more than one population (Imsland et al., 2003; Rolland et al., 2007). Atlantic populations ranging from Denmark to Portugal may be considered one panmictic unit (Exadactylos et al., 1998; Rolland et al., 2007), with weak differentiation between samples from the northern European coast and the British Islands (Exadactylos et al., 2003). Whether or not isolation by distance was observed, depended on the genetic marker applied (Kotoulas et al., 1995; Exadactylos et al., 1998; Rolland et al., 2007; Cuveliers et al., 2012). However, differences in egg size, larval length at hatching, growth and initiation of metamorphosis were observed between progeny of broodstock origination from different geographical regions (Rijnsdorp & Vingerhoed, 1994; Exadactylos et al., 1999). Furthermore, a distinct Mediterranean population was suggested, with separation between the Eastern and Western region (Kotoulas et al., 1995; Garoia et al., 2007; Rolland et al., 2007).

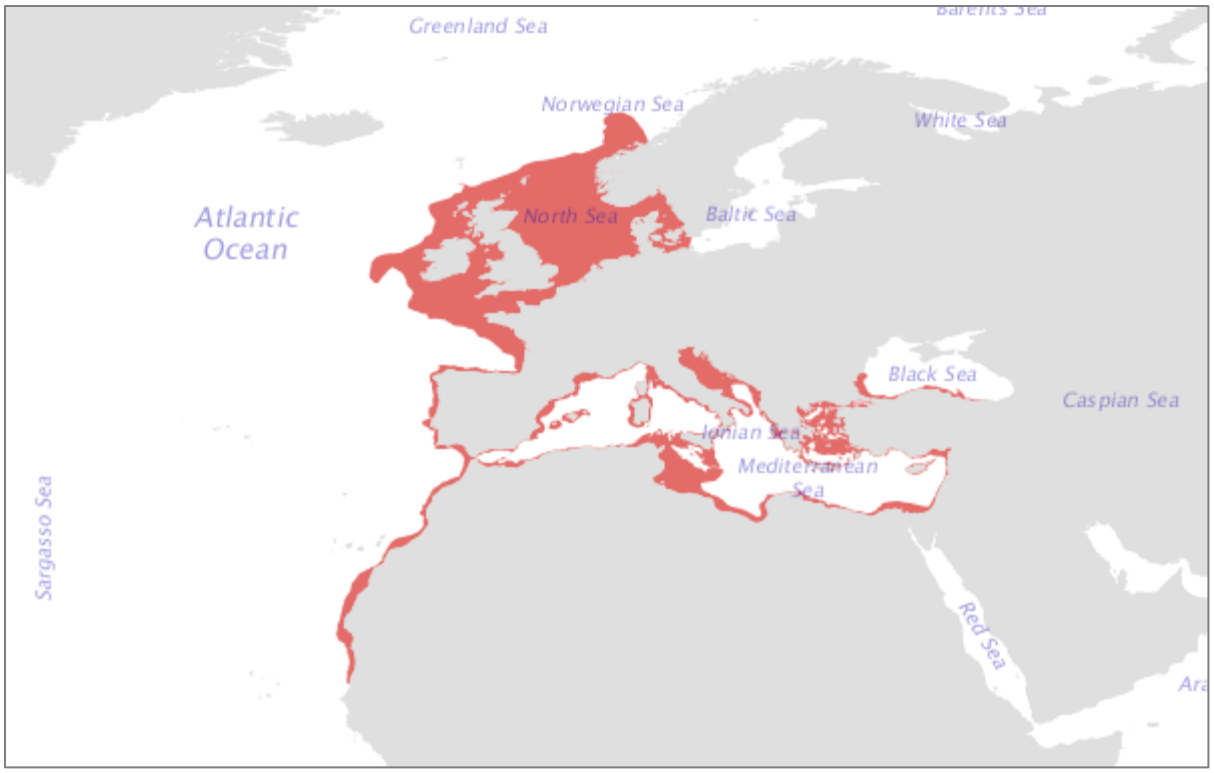


Figure 1.3 - Geographical distribution of Solea solea *(FAO, 2018).*

1.2.2 Life cycle

Adults spawn in shallow coastal waters, except in the Bay of Biscay where spawning takes place between 70 and 90 m depth (Guillou, 1978; Dorel et al., 1991). Sole spawns between December and June, with spawning peaks in the North Sea from April to June (FAO, 2018). The pelagic larva hatches at about 3 mm with unpigmented eyes and may be easily identified by the appearance of the oil globules during the yolk sac stage (Nichols, 1976). Metamorphosis involves a 90° rotation of the body and the migration of one eye to the ocular side (Fernandez-Diaz et al., 2001), together with ossification and skeletal development (Brewster, 1987; Wagemans & Vandewalle, 2001) and is heavily dependent on endocrine regulation (Tveiten & Tromsø, 2009). Asymmetry begins at about 8-9 mm and complete bottom adaptation is reached at 12 mm (Nichols, 1976; Fonds, 1979). These changes are accompanied by the transition from pelagic to a benthic life and consequently large changes in diet (Fernandez-Diaz et al., 2001).

1.3 DOVER SOLE - CAPTURE FISHERIES

The Dover sole market is dominated by the capture fisheries production, which exceeded 65 000 tonnes yearly in the 1990s and fluctuated around 35 000 tonnes in the last two decades (FAO, 2018). Dover sole is harvested primarily in the North Sea, with Subarea IV ('main North sea') being by far the most important fishing ground. To manage the fish stock, a two stage plan for sole was established by

the European Union (Council Regulation EC No. 509/2007). First, a recovery plan was put in place to ensure the return of the stock within safe biological limits. The end of this stage will be announced shortly, as sole stock has been within these limits since 2012 (ICES, 2018). Secondly, in the next stage the sole stock will be managed on the basis of Maximum Sustainable Yield (MSY). Furthermore, the sole fisheries is regulated by annual total allowable catch quota and effort as well as technical restrictions. Sole is harvested by several gear types with beam trawling being the most important. Minimum landing size is 24 cm and minimum mesh size for beam trawlers is 80 mm. Between 2014 and 2017, pulse trawls are used to a growing extent in the flatfish beam trawl fleet in the North Sea, substituting more and more traditional beam trawls. The reduced soil displacement of pulse trawls allows fishing on softer grounds in the North Sea, changing the spatial distribution of the main fisheries to the southern part of subarea IV (ICES, 2018). However, in 2019, the EU parliament has decided to ban pulse trawling starting from 1 July 2021, with a direct halving of the fleet this year (www.europarl.europa.eu). In the Mediterranean, Dover sole is part of a multi-species multi-gear fisheries and catches are relatively small but have increased significantly in the last two decades (Bjørndal et al., 2015).

1.4 DOVER SOLE - AQUACULTURE

In contrast to the capture fisheries, Dover sole aquaculture production is limited and has reached between 30 and 40 tonnes yearly in the 1980s but decreased below 30 tonnes in the following decade. Since 2001, production increased slowly but varied between 9 tonnes (2006) and 158 tonnes (2013) with large annual fluctuations (FAO, 2018). This recent increase in sole farming might be linked to an increased investment in research and development of both Dover and Senegalese sole. Successful Dover sole aquaculture is hampered by the limited knowledge on the basic biology of the species. During the production life cycle, the highest mortality rates still occur in the larvicultural phase, in particular during transition from exogenous to endogenous feeding, weaning and metamorphosis (Bonaldo et al., 2011). Whereas an overwhelming amount of recent literature is available focusing on the biology and aquaculture of Senegalese sole (Bjørndal et al., 2015), publications concerning Dover sole are much more limited.

In what follows, an overview of the status and challenges in Dover sole larviculture is given, since the latter is the topic of this PhD research. First, the larval production is discussed, followed by the main developmental problems observed and diseases encountered, with a focus on disease prevention and the available research tools to investigate this.

1.4.1 Production

1.4.1.1 Spawning and egg production

In aquaculture settings, spawning of Dover sole happens in a wide range of conditions with variable fertilization rates between 20 to 80% (Howell, 1997). Spawning takes place in the narrow temperature range of 8-12 °C and day lengths of 11-16 h of light and responds to photoperiod as well as thermal stimulation (Devauchelle et al., 1987). The eggs are collected at the water outlet and transferred to incubator tanks. In the hatcheries of most fish species, egg disinfection is commonly employed as a mortality mitigation and disease management tool (De Swaef et al., 2016). However, at the initiation of the current PhD research, to our knowledge, no such protocol was available for Dover sole eggs. Broodstock animals spawn naturally during several weeks, making it challenging to obtain high numbers of eggs on a daily basis. However, since cannibalism in larvae is not reported, eggs of several subsequent days may be mixed (Palazzi et al., 2006). Hormonal induction is not routinely performed and although generally applied in other flatfish species, stripping seems difficult in Dover sole and the closely related Senegalese sole, resulting in low fertilization success (Imsland et al., 2003). However, artificial fertilization in Senegalese sole has been studied extensively and promising protocols were published (Rasines et al., 2013). The pelagic egg has a diameter between 1.1 and 1.6 mm with decreasing egg size during the spawning season (Houghton et al., 1985; Rijnsdorp & Vingerhoed, 1994; ICES FishMap, 2018). The optimal egg incubation temperature varies between 13-15 °C which is slightly higher than the spawning temperature (Devauchelle et al., 1987). Nonetheless, temperatures ranging between 10-16 °C also result in normal survival and development (Fonds, 1979). Ideal salinity for eggs and embryos is between 20-35 ppt (Fonds, 1979; Devauchelle et al., 1987). Incubation until hatching is approximately 3.5 days at 17 °C (Fonds, 1979; Devauchelle et al., 1987). Although the macroscopical and microscopical structure are well described for Dover sole eggs (Devauchelle et al., 1987; Munk & Nielsen, 2005), no information on the ultrastructural morphology was available prior to the current PhD research.

Predicting egg quality at an early stage would avoid economic losses but appears to be difficult. In accordance with many marine fish species (Kjørsvik et al., 1990; Anguis & Canavate, 2005), egg quality in Dover sole is thought to be related to the floating capacity of the egg in seawater whereby floating eggs are depicted as good quality and fertilized (Dinis, 1982; Imsland et al., 2003). Moreover, also symmetry of the early blastomeres and transparency of the egg as well as distribution of lipid globules were suggested to be related to quality both in Dover sole (Dinis, 1982) as well as in several other marine species (reviewed in Kjørsvik et al., 1990).

1.4.1.2 Production of juveniles

Before transformation into a more robust juvenile, most fish go through a vulnerable larval phase following the protected embryonic stage inside the egg shell. Many of the problems related to juvenile fish production in aquaculture are linked to the larval stage.

1.4.1.2.1 First-feeding larvae

Following hatching, the pelagic Dover sole larvae grow the first day without feeding, solely relying on their yolk sac containing the vitulus and oil globules. The yolk sac decreases gradually until complete resorption is reached at 15 days after hatching (DAH) (11 °C) (McGurk, 1984). First feeding starts when the mouth opens at approximately 2 (18 °C) to 4 DAH (16 °C) (Fonds, 1979; Devauchelle et al., 1987; Richard et al., 1991) (Figure 1.4). The nutritional requirements of marine fish larvae in general were extensively studied and the most commonly used prey in larval fish culture are *Artemia* and rotifers (Ohs et al., 2012). Also for Dover sole, feeding is based on live food in the initial larval stage. First feeding larvae are characterized by a rudimentary digestive system with a non-functional stomach and low digestive enzymes activity, making it challenging to process and assimilate a complex microdiet (Kolkovski, 2001). Especially in Dover sole, proteolytic digestive enzymes are detected later during gastrointestinal development as compared with for example the closely related Senegalese sole (Clark et al., 1986; Boulhic & Gabaudan, 1992; Ribeiro et al., 1999). In the lab, a diet on freshly-hatched *Artemia* nauplii is sufficient to rear first feeding larvae (Howell, 1997; Lund, 2007). Although the knowledge on nutrient requirements for Dover sole larvae is very limited, it is suggested that the dietary requirement for (n-3) polysaturated fatty acids is low (Howell & Tzoumas, 1991). This is in accordance with Senegalese sole (Morais et al., 2016) but appears to be less strict than for other marine fish larvae (Howell & Tzoumas, 1991). Use of prior enrichment or smaller food organisms (rotifers) does not increase larval survival (Howell, 1973; Fuchs, 1982; Lund, 2007). However, *Artemia* enriched with eicosapentaenoic acid (EPA) had a positive impact on *in vivo* tolerance for hypoxia in Dover sole larvae (McKenzie et al., 2008), presumably related to a more balanced essential fatty acids diet.Furthermore, co-feeding of larvae with preserved copepods resulted in increased growth and survival as well as increased tolerance to stressors (captivity conditions, thermal or density stress-test) (Piccinetti et al., 2012). In addition, young larvae may have specific arachidonic acid (ARA) requirements, as observed for the closely related Senegalese sole (Mourente & Vasquez, 1996).

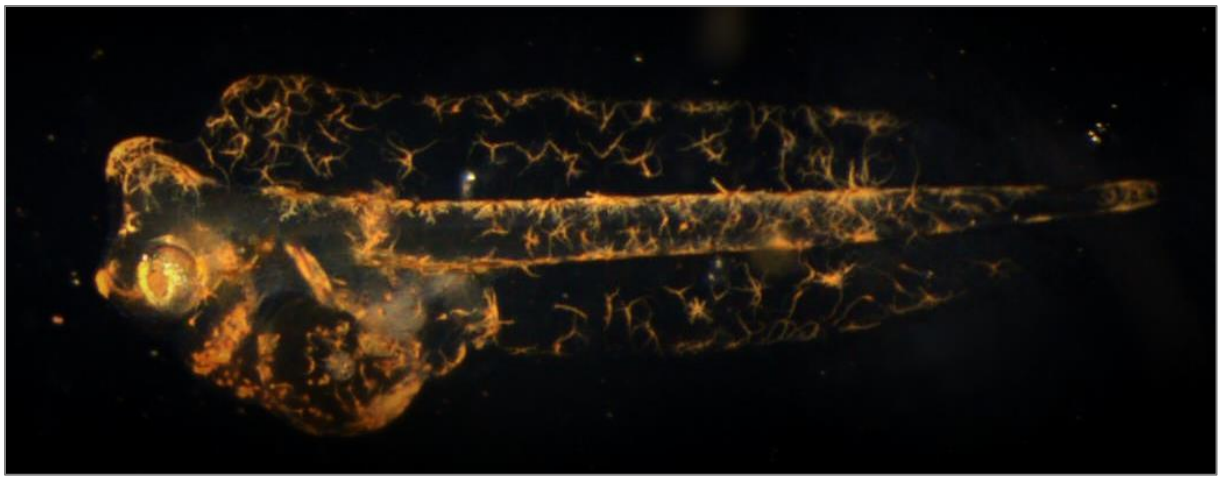


Figure 1.4 - Dover sole first feeding larva with partially resorbed yolk sac (3 DAH).

1.4.1.2.2 Weaning

The use of live feed involves several disadvantages; in the past, *Artemia* cysts were not consistently available (Lavens & Sorgeloos, 2000; Callan et al., 2003)and in this period the declined yields of *Artemia* cysts from the Great Salt Lake also became a topic of concern (Lavens & Sorgeloos, 2000; Piccinetti et al., 2012). However, strict regulations and monitoring ensure sustainable harvesting nowadays (Belovsky & Perschon, 2019) Next, live feed typically contains high bacteria loads, including pathogenic bacteria such as *Vibrio* spp., increasing the risk for larval diseases (Olsen et al., 2000; Makridis et al., 2001; Olafsen, 2001). Total replacement of live feed is not feasible yet, but early weaning using a microdiet might improve performances and increase the competitive potential of Dover sole juvenile production. Co-feeding with microdiets gradually weans larvae off live prey and promotes digestive maturation at early age (Engrola et al., 2007; 2009), improving growth and not restricting survival or metamorphosis of fish larvae (Rosenlund et al., 1997).

In the past, weaning was considered one of the main obstacles of Dover sole rearing and was only initiated at the end of metamorphosis (Bromley, 1977; Day et al., 1997). However, many attractive weaning diets were developed (reviewed in Imsland et al., 2003) and recent studies agree that early weaning is feasible in Dover sole larvae starting from 13 DAH (Bonaldo et al., 2011; Parma et al., 2013) with a limited impact on growth and maintaining normal metamorphosis and survival. Furthermore, this strategy would reduce production costs and improve tank hygiene. The reduced costs associated with a decreased live feed period may compensate the fish farmer for the delay in growth (Parma et al., 2013). Although part of the larvae survive, rearing Dover sole larvae on inert diets starting from mouth opening, severely reduces survival and growth (Appelbaum, 1985; Parma et al., 2013).

1.4.1.2.3 Metamorphosis

Generally, the start of metamorphosis in Dover sole larvae is seen around 13 DAH (18°C) (Ferraresso et al., 2013). In flatfish, metamorphosis is highly complex, including extensive morphological and physiological changes (Figure 1.5), with successful completion being crucial to reliably control the juvenile quality and production quantity. Initiation and advance of metamorphosis are thought to be related to body length and starts when larvae reach approximately 8 mm (Fernandez-Diaz et al., 2001; Lund, 2007). This is in contrast to other flatfish species such as Senegalese sole for which nutritional factors (e.g. EPA) are suggested as the main causative agents (Villalta et al., 2005). High variability in timing of both the start of metamorphosis, characterized by eye migration, as well as caudal metamorphosis was observed (Palazzi et al., 2006). Both steps generally require 8-14 days to reach completion. To characterize the process of metamorphosis, different stages are delineated in the literature. Depending on the literature source, stages of metamorphosis in Dover sole larvae are based on the classification of larval turbot (Al-Maghazachi & Gibson, 1984) or Senegalese sole (Fernandez-Diaz, 2001; Padros et al., 2011) as hitherto no species specific classification scheme is available for Dover sole.

The recent introduction of functional genomics and proteomics in flatfish research enhances the knowledge of the biology of these species and sheds light on the molecular mechanism underlying different physiological processes in amongst others Senegalese sole (Cerda et al., 2008; Darias et al., 2012) and Atlantic halibut (Douglas et al., 2007; Murray et al., 2010). Moreover, characterization of the transcriptome of Dover sole during larval development and metamorphosis was established (Ferraresso et al., 2013; Benzekri et al., 2014). This transcriptome analysis defines a large amount of molecular pathways and gene networks that might play a crucial role in not only organogenesis and larval growth but also the completion of Dover sole metamorphosis (Ferraresso et al., 2013).

Figure 1.5 - Dover sole larva with completed metamorphosis (36 DAH).

1.4.1.3 Main developmental problems

1.4.1.3.1 Skeletal anomalies

The presence of skeletal anomalies is a persisting problem in aquaculture with a high economic impact. Although frequently studied, still a variety of causes are being discussed today with genetic factors (Boglione et al., 2013a), inadequate nutrition (Cahu et al., 2003; Fjelldal et al., 2010) and unsuitable rearing conditions (Fjelldal et al., 2004; Cobcroft & Battaglene, 2009) as the most important factors. Although skeletal anomalies may develop at different moments in time (Witten et al., 2005), most skeletal malformations originate during chondrification and ossification processes at early life stages (Boglione et al., 2013a). Hatchery reared fish present a higher degree of skeletal anomalies compared with wild specimens, indicating an effect of the cultivation practices or a selective mortality of deformed wild fish (e.g. Gavaia et al., 2009). Large variations in the incidence of skeletal anomalies in aquaculture settings are observed between species, rearing conditions and batches. Today, 20% of severe deformities in marine fish farming at the end of the hatchery phase is considered to be normal (Boglione et al., 2013a; 2013b). However, in Senegalese sole, deformity rates up till 80% were reported (Engrola et al.,2009; Boglino et al., 2012). Typical skeletal malformations are situated at the vertrebal column and the head region. In flatfish, vertebral anomalies were reported for juveniles of Senegalese sole (Gavaia et al., 2002; 2009; Fernandez et al., 2009) and Atlantic halibut (Lewis-McCrae & Lall, 2010). Furthermore, skull anomalies due to incorrect migration of the eye or cranial structures (Schreiber, 2006; Gavaia et al., 2009) as well as jaw anomalies (Estevez & Kanazawa, 1995; Saele et al., 2004; Blanco-Vives et al., 2010) during the extensive metamorphosis of flatfish were frequently observed. For Dover sole, a high degree of malformations (up to 48%) on the oral jaw apparatus and teeth

development at the ocular side were noted in laboratory conditions in post-metamorphosis larvae (Lagadère et al., 1993). This appears to be in contrast with the closely related Senegalese sole, where the head region is less affected by anomalies (Morais et al., 2016). Although the cranial development and eye migration are well described during larval development, including metamorphosis for Dover sole (Brewster, 1987; Wagemans & Vandewalle, 2001), to our knowledge, no additional data on skeletal anomalies in Dover sole larvae or juveniles are available. However, considering the large amount of studies focusing on skeletal anomalies in other cultured fish species (reviewed in Boglione et al., 2013b) and the observed malformations in the closely related Senegalese sole (Gavaia et al., 2001; 2009; Fernandez et al., 2009; Boglino et al., 2012; Boglione et al., 2013a) underreporting and limited research may lie at the root of these low figures in Dover sole larviculture. In Senegalese sole, skeletal deformities are linked to dietary requirements. High levels of ARA, especially for larvae prior to metamorphosis, result in a high incidence of skeletal deformities and less mineralized skeletons (Boglino et al., 2012). In addition, high amounts of ARA cause disturbed eye migration and cranial bone remodeling (Boglino et al., 2013). With respect to vitamins, a rise in the number of vertebral deformities is observed following increased dietary levels of vitamin A (Fernandez & Gisbert, 2011), whereas supplementation with vitamin K reduced the amount of malformations (Richard et al., 2014). To our knowledge, the importance of these componounds in the Dover sole larval diet was not yet investigated.

1.4.1.3.2 Malpigmentation

Malpigmentation is one of the most frequent but highly unpredictable abnormalities in cultured flatfish and results in undesirable juveniles for future cultivation (Imsland et al., 2006). Furthermore, highly malpigmented juveniles have a slower growth rate (Lund, 2007). Development of proper pigmentation occurs prior to metamorphosis and is deeply influenced by the larval diet (Reitan et al., 1994 (turbot); Shields et al., 1999 (halibut); Bolker & Hill, 2000; Lund et al., 2010 (sole)). Usually two types of malpigmentation are defined: excessive pigmentation or hypermelanosis (Figure 1.6a) or deficient pigmentation or hypomelanosis (Figure 1.6b) (Bolker & Hill, 2000). Although docosahexaenoic acid (DHA) enrichment was frequently reported to improve pigmentation success in flatfish (e.g. Reitan et al., 1994), no influence of DHA on Dover sole pigmentation was observed (Lund, 2007). Also EPA is regularly mentioned to enhance pigmentation but did not influence pigmentation success in Dover sole (Lund, 2007). However, *Artemia* enriched with high levels of ARA did result in a higher abundance of malpigmentation in Dover sole, in accordance with Senegalese sole (Villalta et al., 2005; Boglino et al., 2013), turbot and Atlantic halibut (McEvoy et al., 1998). The sensitivity to ARA treatment was higher pre-metamorphosis and varied throughout different stages of larval development (Lund, 2007). In Senegalese sole, it was revealed that ARA has an impact on

chromatophore differentiation and abundance during and post metamorphosis, combined with a degradation of the existing xanthophores during the same period (Darias et al., 2013). Furthermore, although to a lesser extent, abiotic factors such as tank colour and light intensity can affect the degree of pseudoalbinism in Dover sole (Lund et al., 2010).

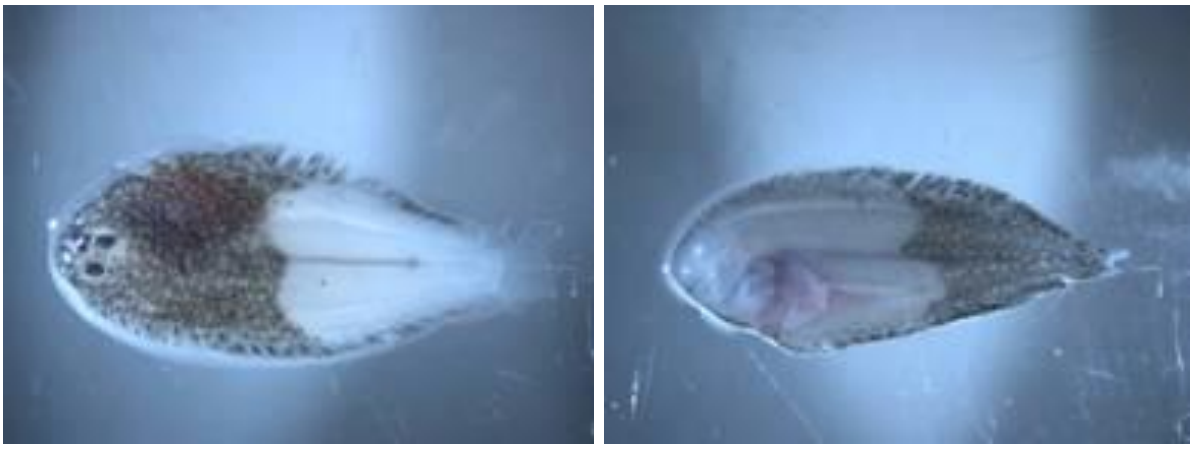


Figure 1.6 - Illustration of hypomelanosis (a) and hypermelanosis (b) in sole juveniles (retrieved from Lund, 2007).

1.4.2 Diseases

In general, sole species (both Senegalese sole and Dover sole) are susceptible to bacterial diseases commonly affecting other cultured flatfish and the severity of the outbreak is mostly linked to the increasing intensification of the larval production. The three main disease problems affecting Dover sole are black patch necrosis, vibriosis and photobacteriosis (FAO, 2018).

In the past, black patch necrosis caused by *Tenacibaculum maritimum,* was reported to be highly infectious in Dover sole aquaculture (Bernadet et al., 1990; Baynes & Howell, 1993). However, combining adequate nutrition and strict hygiene measures appears to be sufficient to avoid black patch necrosis outbreaks (Baynes & Howell, 1993).

Vibriosis, caused by different bacterial species of the *Vibrio* genus, is a challenging bacterial disease affecting both adult and early life stages of fish (Austin & Austin, 2012) with the highest incidence occurring in larval and juvenile stages of marine species (Toranzo, 2004; 2005). Furthermore, many studies stress the importance of pathogenic *Vibrio* species in hatcheries and their ability to generate disease (Touraki et al., 2012; D'Alvise et al., 2013; Silva et al., 2014). Within the genus *Vibrio*, one of the species causing the most (economically) serious diseases in marine culture is *Vibrio anguillarum* (Toranzo, 2005). *V. anguillarum* was isolated in Dover sole (both adults and juveniles) (Manfrin et al., 2003; Paolini et al., 2010) and outbreaks in larvae were reported (Palazzi et al., 2006). Both *V. anguillarum* serovar O1 (Manfrin et al., 2003) as well as serovar O2 (Paolini et al., 2010) were identified

as the causative agents in Dover sole. As has been observed in other flatfish (Olsson et al., 1998), the gut may act as a portal of entry for the bacteria (Paolini et al., 2010).

Photobacteriosis is caused by the halophilic bacteria *Photobacterium damselae* subsp. *piscicida* and has resulted in economic losses in sole aquaculture starting from 1990 in the Mediterranean countries (FAO, 2018). Severe mortalities especially are noted at water temperatures above 18-20 °C (Toranzo, 2004). The majority of the outbreaks takes place during the larval and fingerling stages, impeding standard vaccination programs (Zorilla et al., 1999; Magariños et al., 2003).

This scarce information on Dover sole health management is largely rooted in the fact that Dover sole aquaculture was initiated only recently, underscoring the need for research on health and disease in this alternative aquaculture species.

1.4.2.1 Disease prevention

Disease outbreaks result in large economic losses and prevention is therefore of major importance for the industry (Verscheure et al., 2000). In general, bacterial fish pathogens are considered the most important infectious microbes in aquaculture (Meyer, 1991) and in the past, antibiotics were frequently applied to prevent and treat infectious diseases in larviculture. However, the intensive use of antibiotics as prophylactic treatment in hatcheries was criticized (Skjermo & Vadstein, 1999; Romero et al., 2012). To achieve microbial control without the use of antibiotics, a combination of different strategies was put forward (Vadstein, 1997). A non-selective reduction of bacteria can reduce the outbreak of disease within one hatchery as well as restrict the transition to another aquaculture facility. Therefore, strict hygiene measures are implemented, including egg disinfection and the limitation of organic matter. Furthermore, multiple environmental-friendly prophylactic disease treatments were pinpointed for marine fish larvae, including the use of immunostimulants such as probiotics (Nayak et al., 2010; De et al., 2014), prebiotics (Ringø et al., 2010) or synbiotics (Ringø et al., 2014a). Although valuable in adult fish (e.g. Ringø et al., 2014b), vaccination at this early stage is not possible due to the underdeveloped immune system of the larvae.

1.4.2.1.1 Egg disinfection

Egg disinfection is a widely used mortality mitigation tool in commercial hatcheries as mortality may be induced by high bacterial densities in the rearing tanks, thereby causing overgrowth of the fish egg (Hansen & Olafsen, 1989; Morrison et al., 1999) or asphyxiation of the fish embryo due to oxygen deficiency (Brown et al., 2005). Furthermore, infection with a pathogenic agent can decrease survival and negatively impact the development of the fish embryo and larvae (Munro et al., 1995; Olafsen, 2001; Sorensen et al., 2014). Fish eggs harbouring pathogenic agents also form a major route of disease transmission within and between hatcheries (Peeler et al., 2011). Consequently, various surface egg

disinfection protocols were developed. Multiple chemical disinfectants were evaluated for their bactericidal, virucidal and fungicidal effects and many different protocols were tested as large interspecies differences in tolerance were observed (e.g. Salvesen et al., 1997). For marine flatfish, research on egg disinfection protocols is limited to Atlantic halibut (Harboe et al., 1994; Salvesen et al., 1997), European plaice (*Pleuronectes platessa*, Salvesen et al., 1991), turbot (Salvesen et al., 1997; Grotmol et al., 2003), California flounder *Paralichthys californicus* (Stuart et al., 2010) and spotted halibut *Verasper variegatus* (Hirazawa et al., 1999). Disinfection protocols involve the use of glutaraldehyde (Salvesen et al., 1991; 1997; Harboe et al., 1994), ozone (Grotmol et al., 2003), iodine (Hirazawa et al., 1999; Stuart et al., 2010) and formaldehyde (Stuart et al., 2010). To our knowledge, no egg disinfection protocol was developed for Dover sole prior to the current PhD research nor were there any data on the chemical toxicity of different disinfectants to Dover sole eggs and larvae available. Furthermore, there was a clear lack of a critical review listing the large variety of studies implementing different species and products. Such a review would help in making a well-founded choice in the chemicals and protocols included in egg disinfection research involving new fish species.

1.4.2.1.2 Probiotics

Probiotics are usually defined as 'products which contain viable non-pathogenic micro-organisms able to confer health benefits to the host'. In aquaculture practices, probiotics became an integral part of the culture practices to improve growth and disease resistance (Nayak, 2010; De et al., 2014). A variety of benefits were linked to probiotic treatment, ranging from increased feed conversion (Al-Dohail et al., 2009; De Rodriganez et al., 2009) and decomposition of organic matter (Boyd & Massaut, 1999) to increased larval protection against pathogens. For the latter, several processes may be involved. Competitive exclusion for adhesion sites in the larval gut (Chabrillon et al., 2005; Vine et al., 2006), as well as the production of antagonistic substances such as organic acids, hydrogen perioxide, antibiotics and lysozymes (Sugita et al., 1998; El-Dakar et al., 2007) were linked to the protective potential of probiotics. The most commonly alleged benefit of probiotics is modulation of the immune system (reviewed in Nayak, 2010; De et al., 2014) by enhancing both systemic as well as local gut immunity of the fish. Research concerning the effect of probiotic supplementation on disease resistance of marine flatfish is limited to turbot (De Schrijver & Ollevier, 2000; Hjelm et al., 2004), Japanese flounder (*Paralichthys olivaceus,* Taoka et al., 2006), Senegalese and Dover sole (summarized in Table 1.1). Probiotic supplementation resulted in better food conversion in juveniles (De Schrijver & Ollevier, 2000) as well as increased survival of turbot larvae, whether or not following pathogenic challenge (Ringø & Vadstein, 1998; Gatesoupe, 1997; Hjelm et al., 2004; Huys et al., 2001; Planas et al., 2006). Addition of a probiotic mixture to Japanese flounder juveniles resulted in increased survival and decreased mortality following challenge (Taoka et al., 2006). Until now, two probiotics, *Shewanella*

putrefaciens pdp11 and *S. baltica* pdp13 were isolated for Senegalese sole resulting in improved growth and performance in both juveniles (Diaz-Rosales et al., 2009; de la Banda et al., 2010; Tapia-Paniagua et al., 2014) and larvae (Makridis et al., 2008; Lobo et al., 2014; Jurado et al., 2018) following supplementation. For Dover sole larvae, probiotic *Enterococcus faecium* IMC511 was reported to result in increased survival and a positive impact on the neuroendocrine system (Avella et al., 2011; Palermo et al., 2011). Hitherto, no probiotic protection following pathogenic challenge was described in sole larvae.

1.4.2.1.3 Prebiotics

Prebiotics were defined as 'nonviable food components that confer a health benefit on the host associated with modulation of the microbiota' (FAO, 2018). Supplementation of the fish diet with prebiotics can potentially enhance the innate immunity of the fish, hereby improving survival following pathogen exposure (Bricknell & Dalmo, 2005; Ringø et al., 2012). In adult fish, such beneficial effects were frequently observed in different fish species and a variety of chemical components are suggested. The most common prebiotics used in fish are oligosaccharides, in particular mannan oligosaccharide (MOS) and fructooligosaccharides (FOS), but recently also inulin and high M-alginates have become increasingly important. Several general reviews were published over the past decade summarizing the available literature (e.g. Burr et al., 2005; Merrifield et al., 2010; Dimitroglou et al., 2011; Ringø et al., 2012; 2014). Publications on marine flatfish are limited and comprise turbot, halibut and one study on Senegalese sole (Table 1.2). Four different prebiotics (chitosan, FOS, inulin and high M-alginates) were tested in turbot juveniles with overall positive effects on larval health and survival (Table 1.2). For turbot larvae only alginate was tested, resulting in increased protein synthesis and turnover (Conceicao et al., 2001). Following laminaran and high-M alginate supplementation, increased survival and growth were observed in Atlantic halibut larvae (Vadstein et al., 1993; Strand & Dalmo, 1997; Skjermo & Bergh, 2004). Furthermore, increased survival following pathogen challenge was observed in juveniles of Senegalese sole supplemented with MOS (Dimitroglou et al., 2011). To our knowledge, no prebiotic treatments for Dover sole and the closely related Senegalese sole larvae were reported.

1.4.2.1.4 Synbiotics

Synbiotics are defined as a nutritional supplements combining prebiotics and probiotics in a synergistic way, resulting in beneficial effects on the host health and survival (Andersson et al., 2001). Although beneficial effects were reported, the amount of studies concerning synbiotics are limited and the use of these combinations in fish farms hitherto poorly investigated (Cerezuela et al., 2011). For marine flatfish, symbiotic applications are limited to one study on Japanese flounder juveniles. Combining the prebiotic components MOS and FOS with the probiotic *Bacillus clausii* resulted in increased body

weight and digestive enzyme activity (Ye et al., 2011). To our knowledge, no information on the effect of synbiotics on flatfish larvae, including Dover sole, are available.

1.5 RESEARCH TOOLS IN LARVAL STUDIES

The above mentioned hindrances in Dover sole larviculture and in particular the very limited knowledge on this species, stress the need for increased research efforts on the larval production phase. In this respect, a higher reproducibility as well as substantiated comparison between studies is to be expected with the use of a standardized and controlled experimental set-up. In view of this, three important research tools will be elaborated on.

1.5.1 Multi well plate housing system

During larval research, the animals are mostly group housed in tanks, small beakers or wells, depending on the amount of larvae. Concerning Dover sole, most experiments are performed in tanks (Fonds, 1979; Exadactylos et al., 1999; Avella et al., 2011; Bonaldo et al., 2011; Palermo et al., 2011; Ferraresso et al., 2013). In these cases, larvae are housed in large groups, mimicking the set-up in commercial hatchery and rearing facilities. Water exchange is (semi) automatic in this systems, limiting the amount of work needed to maintain the animals. Traditional rearing tanks have limited possibilities to control the microbiota, both the transmission of bacteria in or out the system as well as bacterial (over) growth in the tanks (Yufera, 2018). Alternatively, to monitor Dover sole larvae following exposure, small seawater bodies containing 20-25 larvae/beaker are implemented (Foekema et al., 2008; Bolle et al., 2012; Desender et al., 2018). The latter experimental set-up requires labour-intensive manual water renewals if larvae are maintained for longer periods. One study group housed the Dover sole larvae in well plates following treatment (de Boer et al., 2012). In this study, larvae were kept in six-well plates for seven days, each well containing five larvae. Larvae were not fed and no water exchanges were performed. Regardless of the housing system, group housing may result in large variations between replicates (Bolle et al., 2012; Situmorang et al., 2014) which may be explained by the death of one larva impacting the health of the other larvae housed in the same system. Decomposition of dead larvae threaten the health of the co-housed larvae by reducing the water quality through decreased oxygen concentrations and increased ammonia levels (Goncalves & Gagnon, 2011; Wang et al., 2015). Furthermore, the reproducibility of pathogenesis studies may be hampered as (sterile) dead larvae present in rearing vials were reported to change the virulence of the pathogens (Li et al., 2014). As a result, the last two decades more and more researchers have made efforts in developing an experimental set-up in which the fish larvae may be held individually in multi well plates. This technique ensures that the possible death of one larva has no effect on the adjacent one. Implementing this system also facilitates monitoring of the health status and behavior of the individually housed larvae. Furthermore, the wells are considered independent replicates for statistical

analyses (OECD, 2013), reducing the number of experimental animals needed. With regard to larval flatfish, a multi well plate housing systems was developed and implemented for turbot (Planas et al., 2006; Rønneseth et al., 2017), halibut (Rønneseth et al., 2017) and Senegalese sole (Makridis et al., 2008; Martin et al., 2014) but prior to this study, no information on the feasibility of a multi well housing system for Dover sole was available.

1.5.2 Gnotobiotic model

To enhance Dover sole larviculture, improving disease prevention is of uttermost importance. The use of animals raised in gnotobiotic conditions was suggested to be an excellent tool to evaluate the interaction between microbial communities and the larval host, hereby assessing the possible impact on larval health as well as understand the pathology (Marques et al., 2005; Pham et al., 2008; Dierckens et al., 2009). The word 'gnotobiotic' is derived from the Greek words 'gnotos' (knowledge) and 'biota' (life), and refers to an environment in which all microorganisms are either defined or excluded (Pham et al., 2008).

Furthermore, these gnotobiotic models are highly useful in evaluating new methods for disease control. Although pro- and prebiotics are widely studied and many suggestions on their mode of action were made, the actual factors that determine their immunomodulatory properties are not known in marine fish (larvae) (Nayak, 2010; Ringø et al., 2014). In conventional experiments, interaction between the immunostimulants and the (unknown) microbial community present, hampers determination of the specific mode of actions. Hence, gnotobiotic models may be instrumental in understanding the basic mechanism of the pre and probiotic activity (Nayak, 2010). These models require complete elimination of the bacterial community present, followed by colonization of the animals with specific (combinations of) bacteria. For fish larvae, elimination of the bacteria was established by egg disinfection, followed by incubation of the eggs and hatched larvae in sterile seawater. The number of gnotobiotic larval models is limited due to the delicate balance between sterility and hatchability of the eggs. Until now, considering roundfish larvae, research teams succeeded in obtaining bacteria free zebrafish (*Danio rerio*, Rawls et al., 2004; Pham et al., 2008), Atlantic cod (*Gadus morhua*, Forberg et al., 2011), seabass (Dierckens et al., 2009; Schaeck et al., 2016) and Nile Tilapia (*Oreochromis niloticus*, Situmorang et al., 2014). Gnotobiotic models for flatfish larvae were only established for turbot (Munro et al., 1995) and halibut (Verner-Jeffreys et al., 2003). For the latter two species, the gnotobiotic status was claimed based solely on culture dependent techniques which may give false negative results due to the slow growth or the presence of viable non culturable organisms (Davis, 2014). Recently, more advanced techniques such as denaturing gradient gel electrophoresis (DGGE) and analysis of PCR fragments generated by universal primer analysis or flow cytometry ensure the quantification and identification of these non culturable bacteria in a reliable

and fast manner (Marques et al., 2005; Forberg et al., 2012; Schaeck et al., 2016). Next to egg disinfection, antibiotics were adopted in all studies but one (zebrafish, Rawls et al., 2004) in the hatching or rearing water. Although without negative effects on the hatchability, caution is needed to remove all antimicrobial agents residues before the start of any experiment to avoid unwanted and unknown interactions with the target organism (Marques et al., 2006). The development of a gnotobiotic model for Dover sole larvae, preferably without the use of antibiotics, would signify a step forward in research concerning the host-microbe interactions in this species.

1.5.3 Challenge model

To gather more knowledge on the interaction between host and pathogen and investigate the effectiveness as well as the mode of action of the recently developed prophylactic disease treatments, including pro- and prebiotics, a reliable experimental challenge model is imperative. Pathogenic challenge models involve the deliberate exposure of larvae to a specific infectious agent, resulting in disease or mortality of part of the larvae in a reproducible manner. The induced mortality needs to be adequately high to enable the exploration of the protective effect of prophylactic or curative treatments. In contrast, a challenge model inducing too high a mortality is also not desirable as this would hamper detection of the possible protective potential of the tested components. The number of studies focusing on pinpointing challenge models in fish larvae is limited. Considering flatfish species, experimental challenges were performed for turbot (Munro et al., 1995; Bergh et al., 1997; Ringø & Vadstein, 1998; Planas et al., 2006), Atlantic halibut (Bergh et al., 1997) and Japanese flounder (Muroga et al., 1990) but not for Dover sole.

In conclusion, Dover sole larviculture would benefit from an increased research effort, focusing on the above mentioned drawbacks and including the adaptation of existing research tools in larval studies for Dover sole larvae.

Table 1.1 - Overview of the probiotics used in flatfish aquaculture, indicating the fish species, probiotic strain(s), the developmental stage (larvae/juveniles) and the observed effects.

Species	Probiotic	Larvae/ juveniles	Effect	Reference
Paralichthys olivaceus	*Bacillus subtilis, Lactobacillus acidophilus, Clostridium butyricum* and *Saccharomyces cerevisiae*	juveniles	Increased survival, water quality, decreased mortality following heat stress test and challenge with *V. anguillarum*	Taoka et al., 2006
Scophthalmus maximus	*Vibrio proteolyticus*	juveniles	Increased food conversion	De Schrijver & Ollevier, 2000
	Roseobacter strain 27-4	larvae	Decreased mortality	Hjelm et al., 2004
	Roseobacter strain 27-4	larvae	Decreased mortality following challenge with *V. anguillarum*	Planas et al., 2006
	Strain E (*V. alginolyticus-alike)*	larvae	Decreased mortality following challenge with *Vibrio* sp.	Gatesoupe, 1997
	V. pelagius	larvae	Decreased mortality following challenge with *A. caviae*	Ringø & Vadstein, 1998
	V. mediterranei Q40 strain	Larvae	Increased survival	Huys et al., 2001
Solea senegalensis	*Shewanella putrefaciens* pdp11	juveniles	Increased growth, decreased mortality following challenge with *P. damselae* subsp. *piscicidae*	Garcia de la Banda et al., 2010

		juveniles	Changed composition of fish intestinal microbiota	Tapia-Paniagua et al., 2004
		juveniles	Decreased mortality following challenge with *P. damselae* subsp. *piscicidae*	Diaz-Rosales, 2009
		larvae	Beneficial for larval development	Jurado et al., 2018
		larvae	Increased growth, better food conversion, modulated larval and fry gut microbiota	Lobo et al., 2014
		larvae	Increased survival during the first phase, decreased bacterial load in the gut	Makridis et al., 2008
	S. baltica pdp13	juveniles	Decreased mortality following challenge with *P. damselae* subsp. *piscicidae*	Diaz-Rosales, 2009
Solea solea	*Enterococcus faecium* IMC511	larvae	Decreased Heat Shock Protein 70 levels, changed composition of fish intestinal microbiota	Avella et al., 2011
	E. faecium IMC511	larvae	Modified neuroendocrine system resulting in changed gene expression profiles throughout development	Palermo et al., 2011

Table 1.2 - Overview of the prebiotics used in flatfish aquaculture, indicating the fish species, prebiotic strain (s), the developmental stage (larvae/juveniles) and the observed effects.

Species	**Prebiotic**	**Larvae/ juveniles**	**Effect**	**Reference**
Scophthalmus maximus	Chitosan oligosaccharides	juveniles	Decreased mortality following challenge with *Edwardsiella tarda*	Cui et al., 2012
	FOS and inulin	juveniles	Increased growth, modulation of the gut microbiota	Mahious et al., 2006
	Alginate	larvae	Increased protein synthesis and turnover	Conceicao et al., 2001
	High-M alginates	juveniles	Increased survival	Skjermo et al., 1995
Hippoglossus hippoglossus	B-glucan (laminaran)	larvae	/	Strand & Dalmo, 1997
	High-M alginate	larvae	Increased survival	Vadstein et al., 1993
	High-M alginate	larvae	Increased growth	Skjermo & Bergh, 2004
Solea senegalensis	MOS	juveniles	Decreased mortality following challenge with *P. damselae* subsp. *piscicida* and improved gut morphology	Dimitroglou et al., 2011

1.6 REFERENCES

Al-Dohail, M.A., Hashim, R. & Aliyu-Paiko, M. (2009) Effects of the probiotic, *Lactobacillus acidophilus*, on the growth performance, haematology parameters and immunoglobulin concentration in African Catfish (*Clarias gariepinus*, Burchell 1822) fingerling. *Aquaculture Research,* **40,** 1642-1652.

Al-maghazachi, S.J. & Gibson, R. (1984) The Developmental Stages of Larval Turbot, *Scophthalmus Maximus* (L). *Journal of Experimental Marine Biology and Ecology,* **82,** 35-51.

Andersson, H., Asp, N.-G., Bruce, A., Ross, S.,Wadström, T. & Wold, A.E. (2001) Health effects of probiotics and prebiotics: a literature review on human studies. *Scandinavian Journal of Nutrition*, **45**, 58–75.

Anguis, V. & Canavate, J.P. (2005) Spawning of captive Senegal sole (*Solea senegalensis*) under a naturally fluctuating temperature regime. *Aquaculture,* **243,** 133-145.

Appelbaum, S. (1985) Rearing of the Dover Sole, *Solea Solea* (L), through Its Larval Stages Using Artificial Diets. *Aquaculture,* **49,** 209-221.

Avella, M.A., Olivotto, I., Silvi, S., Ribecco, C., Cresci, A., Palermo, F., Polzonetti, A. & Carnevali, O. (2011) Use of *Enterococcus faecium* to improve common sole (*Solea solea*) larviculture. *Aquaculture,* **315,** 384-393.

Austin, B. & Austin, D.A. (2012) *Bacterial Fish Pathogens: disease of farmed and wild fish*. Fifth edition. Springer Dordrecht, the Netherlands.

Baynes, S.M. & Howell, B.R. (1993) Observations on the growth, survival and disease resistance of juvenile common sole, *Solea solea* (L.), fed *Mytilus edulis* L*. Journal of Aquaculture and Fisheries Management*, **24**, 95–100.

Belovsky, G.E. & Perschon, W.C. (2019) A management case study for a new commercial fishery: brine shrimp harvesting in Great Salt Lake, Utah, USA. *Ecological applications,* **29,** e01864.

Benzekri, H., Armesto, P., Cousin, X., Rovira, M., Crespo, D., Merlo, M.A., Mazurais, D., Bautista, R., Guerrero-Fernandez, D., Fernandez-Pozo, N., Ponce, M., Infante, C., Zambonino, J.L., Nidelet, S., Gut, M., Rebordinos, L., Planas, J.V., Begout, M.L., Claros, M.G. & Manchado, M. (2014) De novo assembly, characterization and functional annotation of Senegalese sole (*Solea senegalensis*) and common sole (*Solea solea*) transcriptomes: integration in a database and design of a microarray. *Bmc Genomics,* **15**.

Bergh, O., Hjeltnes, B. & Skiftesvik, A.B. (1997) Experimental infection of turbot *Scophthalmus maximus* and halibut *Hippoglossus hippoglossus* yolk sac larvae with *Aeromonas salmonicida* subsp *salmonicida*. *Diseases of Aquatic Organisms,* **29,** 13-20.

Bernadet, J.F., Campbell, A.C. & Buswell, J.A. (1990) *Flexibacter maritimus* is the agent of ''black patch necrosis'' in Dover sole in Scotland. *Diseases of Aquatic Organisms*, **8**, 233–237.

Bjørndal, T. & Guillen, J. (2014) The future of sole farming in Europe: cost of production and markets. *Aquaculture Europe Magazine*, **39**, 2.

Bjørndal, T., Guillen, J., Imsland, A. & Øiestad, V. (2015) Sole: production and market. *GLOBEFISH Research Programme,* Rome, FAO, **118**, 1-72.

Bjørndal, T., Guillen, J. & Imsland, A. (2016) The potential of aquaculture sole production in Europe: Production costs and markets. *Aquaculture Economics & Management,* **20,** 109-129.

Blanco-Vives, B., Villamizar, N., Ramos, J., Bayarri, M.J., Chereguini, O. & Sanchez-Vazquez, F.J. (2010) Effect of daily thermo- and photo-cycles of different light spectrum on the development of Senegal sole (*Solea senegalensis*) larvae. *Aquaculture,* **306,** 137-145.

Boglino, A., Darias, M.J., Estevez, A., Andree, K.B. & Gisbert, E. (2012) The effect of dietary arachidonic acid during the Artemia feeding period on larval growth and skeletogenesis in Senegalese sole, *Solea senegalensis*. *Journal of Applied Ichthyology,* **28,** 411-418.

Boglino, A., Wishkerman, A., Darias, M.J., Andree, K.B., de la Iglesia, P., Estévez, A. & Gisbert, E. (2013) High dietary arachidonic acid levels affect the process of eye migration and head shape in pseudoalbino Senegalese sole Solea senegalensis early juveniles. *Journal of Fish Biology,* **83**, 1302-1320.

Boglione, C., Gavaia, P., Koumoundouros, G., Gisbert, E., Moren, M., Fontagne, S. & Witten, P.E. (2013a) Skeletal anomalies in reared European fish larvae and juveniles. Part 1: normal and anomalous skeletogenic processes. *Reviews in Aquaculture,* **5,** S99-S120.

Boglione, C., Gisbert, E., Gavaia, P., Witten, P.E., Moren, M., Fontagne, S. & Koumoundouros, G. (2013b) Skeletal anomalies in reared European fish larvae and juveniles. Part 2: main typologies, occurrences and causative factors. *Reviews in Aquaculture,* **5,** S121-S167.

Bolker, J.A. & Hill, C.R. (2000) Pigmentation development in hatchery-reared flatfishes. *Journal of Fish Biology,* **56,** 1029-1052.

Bolle, L.J., De Jong, C.a.F., Bierman, S.M., Van Beek, P.J.G., Van Keeken, O.A., Wessels, P.W., Van Damme, C.J.G., Winter, H.V., De Haan, D. & Dekeling, R.P.A. (2012) Common Sole Larvae Survive High Levels of Pile-Driving Sound in Controlled Exposure Experiments. *Plos One,* **7**.

Bonaldo, A., Parma, L., Bacliani, A., Serratore, P. & Gatta, P.P. (2011) Very early weaning of common sole (*Solea solea* L.) larvae by means of different feeding regimes and three commercial microdiets: Influence on performances, metamorphosis development and tank hygiene. *Aquaculture,* **321,** 237-244.

Boulhic, M. & Gabaudan, J. (1992) Histological Study of the Organogenesis of the Digestive-System and Swim Bladder of the Dover Sole, *Solea Solea* (Linnaeus 1758). *Aquaculture,* **102,** 373-396.

Boyd, C.E. & Massaut, L. (1999) Risks associated with the use of chemicals in pond aquaculture. *Aquacultural Engineering,* **20,** 113-132.

Brewster, B. (1987) Eye Migration and Cranial Development during Flatfish Metamorphosis - a Reappraisal (Teleostei, Pleuronectiformes). *Journal of Fish Biology,* **31,** 805-833.

Bricknell, I. & Dalmo, R.A. (2005) The use of immunostimulants in fish larval aquaculture. *Fish & Shellfish Immunology,* **19,** 457-472.

Bromley, P.J. (1977) Methods of Weaning Juvenile Hatchery Reared Sole (*Solea Solea* (L)) from Live Food to Prepared Diets. *Aquaculture,* **12,** 337-347.

Brown, N., Kling, L. & Skonberg, D. (2005) Final report to NOAA: Demonstration of Sustainable Cod Farming from Egg to Grow-out in Maine. In, p. 55. University of Maine.

Burr, G., Gatlin, D. & Ricke, S. (2005) Microbial ecology of the gastrointestinal tract of fish and the potential application of prebiotics and probiotics in finfish aquaculture. *Journal of the World Aquaculture Society,* **36,** 425-436.

Cahu, C., Infante, J.Z. & Takeuchi, T. (2003) Nutritional components affecting skeletal development in fish larvae. *Aquaculture,* **227,** 245-258.

Callan, C., Jordaan, A. & Kling, L.J. (2003) Reducing Artemia use in the culture of Atlantic cod (*Gadus morhua*). *Aquaculture,* **219,** 585-595.

Cerda, J., Mercade, J., Lozano, J.J., Manchado, M., Tingaud-Sequeira, A., Astola, A., Infante, C., Halm, S., Vinas, J., Castellana, B., Asensio, E., Canavate, P., Martinez-Rodriguez, G., Piferrer, F., Planas, J.V., Prat, F., Yufera, M., Durany, O., Subirada, F., Rosell, E. & Maes, T. (2008) Genomic resources for a

commercial flatfish, the Senegalese sole (*Solea senegalensis*): EST sequencing, oligo microarray design, and development of the Soleamold bioinformatic platform. *Bmc Genomics,* **9**.

Cerezuela, R.,Meseguer, J. & Esteban, M.A. (2011) Current knowledge in symbiotic use for fish aquaculture: a review. *Journal of Aquaculture Research and Development,* **S1**, 008.

Chabrillon, M., Rico, R.M., Balebona, M.C. & Morinigo, M.A. (2005) Adhesion to sole, *Solea senegalensis* Kaup, mucus of microorganisms isolated from farmed fish, and their interaction with *Photobacterium damselae* subsp *piscicida*. *Journal of Fish Diseases,* **28,** 229-237.

Clark, J., Murray, K.R. & Stark, J.R. (1986) Protease Development in Dover Sole [*Solea Solea* (L)]. *Aquaculture,* **53,** 253-262.

Cobcroft, J.M. & Battaglene, S.C. (2009) Jaw malformation in striped trumpeter *Latris lineata* larvae linked to walling behaviour and tank colour. *Aquaculture,* **289,** 274-282.

Conceicao, L.E.C., Skjermo, J., Skjak-Braek, G. & Verreth, J.a.J. (2001) Effect of an immunostimulating alginate on protein turnover of turbot (*Scophthalmus maximus* L.) larvae. *Fish Physiology and Biochemistry,* **24,** 207-212.

Cui, L., Xu, W., Ai, Q., Wang, D. & Mai, K. (2012) Effects of dietary chitosan oligosaccharide complex with rare earth on growth performance and innate immune response of turbot, *Scophthalmus maximus* L. *Aquaculture Research,* **44**, 683–690.

Cuveliers, E.L., Larmuseau, M.H.D., Hellemans, B., Verherstraeten, S.L.N.A., Volckaert, F.A.M., & Maes, G.E. (2012) Multi-marker estimate of genetic connectivity of sole (*Solea solea*) in the North-East Atlantic Ocean. *Marine Biology*, **159**, 15.

D'Alvise, P.W., Lillebo, S., Wergeland, H.I., Gram, L. & Bergh, O. (2013) Protection of cod larvae from vibriosis by *Phaeobacter* spp.: A comparison of strains and introduction times. *Aquaculture,* **384,** 82-86.

Darias, M.J., Boglino, A., Manchado, M., Ortiz-Delgado, J.B., Estevez, A., Andree, K.B. & Gisbert, E. (2012) Molecular regulation of both dietary vitamin A and fatty acid absorption and metabolism associated with larval morphogenesis of Senegalese sole (*Solea senegalensis*). *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology,* **161,** 130-139.

Darias, M.J., Andree, K.B., Boglino, A., Rotllant, J., Cerda-Reverter, J.M., Estevez, A. & Gisbert, E. (2013) Morphological and molecular characterization of dietary-induced pseudo-albinism during post-embryonic development of Solea senegalensis (Kaup, 1858). *PLoS ONE* **8**, e68844.

Davis, C. (2014) Enumeration of probiotic strains: review of culture-dependent and alternative techniques to quantify viable bacteria. *Journal of microbiological methods*, **103**, 9-17.

Day, O.J., Howell, B.R. & Jones, D.A. (1997) The effect of dietary hydrolysed fish protein concentrate on the survival and growth of juvenile Dover sole, *Solea solea* (L.), during and after weaning. *Aquaculture Research,* **28,** 911-921.

De, B.C., Meena, D.K., Behera, B.K., Das, P., Das Mohapatra, P.K. & Sharma, A.P. (2014) Probiotics in fish and shellfish culture: immunomodulatory and ecophysiological responses. *Fish Physiology and Biochemistry,* **40,** 921-971.

De Boer, M.K., Boeree, C., Sjollema, S.B., De Vries, T., Rijnsdorp, A.D. & Buma, A.G.J. (2012) The toxic effect of the marine raphidophyte *Fibrocapsa japonica* on larvae of the common flatfish sole (*Solea solea*). *Harmful Algae,* **17,** 92-101.

De La Banda, I.G., Lobo, C., Leon-Rubio, J.M., Tapia-Paniagua, S., Balebona, M.C., Morinigo, M.A., Moreno-Ventas, X., Lucas, L.M., Linares, F., Arce, F. & Arijo, S. (2010) Influence of two closely related probiotics on juvenile Senegalese sole (*Solea senegalensis*, Kaup 1858) performance and protection against *Photobacterium damselae* subsp *piscicida*. *Aquaculture,* **306,** 281-288.

De Rodriganez, M.S., Diaz-Rosales, P., Chabrillon, M., Smidt, H., Arijo, S., Leon-Rubio, J., Alarcon, F., Balebona, M., Morinigo, M., Cara, J. & Moyano, F. (2009) Effect of dietary administration of probiotics on growth and intestine functionality of juvenile Senegalese sole (*Solea senegalensis*, Kaup 1858). *Aquaculture Nutrition,* **15,** 177-185.

De Schrijver, R. & Ollevier, F. (2000) Protein digestion in juvenile turbot (*Scophthalmus maximus*) and effects of dietary administration of *Vibrio proteolyticus*. *Aquaculture,* **186,** 107-116.

De Swaef, E., Van Den Broeck, W., Dierckens, K. & Decostere, A. (2016) Disinfection of teleost eggs: a review. *Reviews in Aquaculture,* **8,** 321-341.

Desender, M., Dumolein, L., Duchateau, L., Adriaens, D., Delbare, D., Polet, H., Chiers, K. & Decostere, A. (2018) Pulse Trawling: The Impact of Pulsed Direct Current on Early Life Stages of Sole *Solea solea*. *North American Journal of Fisheries Management,* **38,** 432-438.

Devauchelle, N., Alexandre, J.C., Lecorre, N. & Letty, Y. (1987) Spawning of Sole (*Solea Solea*) in Captivity. *Aquaculture,* **66,** 125-147.

Diaz-Rosales, P., Arijo, S., Chabrillon, M., Alarcon, F.J., Tapia-Paniagua, S.T., Martinez-Manzanares, E., Balebona, M.C. & Morinigo, M.A. (2009) Effects of two closely related probiotics on respiratory burst

activity of Senegalese sole (*Solea senegalensis*, Kaup) phagocytes, and protection against *Photobacterium damselae* subsp *piscicida*. *Aquaculture,* **293,** 16-21.

Dierckens, K., Rekecki, A., Laureau, S., Sorgeloos, P., Boon, N., Van Den Broeck, W. & Bossier, P. (2009) Development of a bacterial challenge test for gnotobiotic sea bass (*Dicentrarchus labrax*) larvae. *Environmental Microbiology,* **11,** 526-533.

Dimitroglou, A., Merrifield, D.L., Carnevali, O., Picchietti, S., Avella, M., Daniels, C., Guroy, D. & Davies, S.J. (2011) Microbial manipulations to improve fish health and production - A Mediterranean perspective. *Fish & Shellfish Immunology,* **30,** 1-16.

Dinis, M.T., Ribeiro, L., Soares, F. & Sarasquete, C. (1999) A review on the cultivation potential of *Solea senegalensis* in Spain and in Portugal. *Aquaculture,* **176,** 27-38.

Dorel, D., Koutsikopoulos, C., Desaunay, Y. & Marchand J. (1991) Seasonal distribution of young sole (*Solea solea* (L.)) in the nursery ground of the Bay of Vilaine (Northern Bay of Biscay). *Netherlands Journal of Sea Research*, **27,** 297-306.

Douglas, S.E., Knickle, L.C., Kimball, J. & Reith, M.E. (2007) Comprehensive EST analysis of Atlantic halibut (*Hippoglossus hippoglossus*), a commercially relevant aquaculture species. *Bmc Genomics,* **8**.

El-Dakar, A.Y., Shalaby, S.M. & Saoud, I.P. (2007) Assessing the use of a dietary probiotic/prebiotic as an enhancer of spinefoot rabbitfish *Siganus rivulatus* survival and growth. *Aquaculture Nutrition,* **13,** 407-412.

Engrola, S., Conceicao, L.E.C., Dias, L., Pereira, R., Ribeiro, L. & Dinis, M.T. (2007) Improving weaning strategies for Senegalese sole: effects of body weight and digestive capacity. *Aquaculture Research,* **38,** 696-707.

Engrola, S., Figueira, L., Conceicao, L.E.C., Gavaia, P.J., Ribeiro, L. & Dinis, M.T. (2009) Co-feeding in Senegalese sole larvae with inert diet from mouth opening promotes growth at weaning. *Aquaculture,* **288,** 264-272.

European Environment Agency (2018) Report: Aquaculture production, 1-26.

Estevez, A. & Kanazawa, A. (1995) Effect of (n-3) PUFA and vitamin A Artemia enrichment on pigmentation success of turbot*, Scophthalmus maximus* (L). *Aquaculture Nutrition,* **1,** 159-168.

Exadactylos, A., Geffen, A.J. & Thorpe, J.P. (1998) Population structure of the Dover sole, *Solea solea* L., in a background of high gene flow. *Journal of Sea Research,* **40,** 117-129.

Exadactylos, A., Geffen, A.J. & Thorpe, J.P. (1999) Growth and genetic variation in hatchery-reared larval and juvenile Dover sole, *Solea solea* (L.). *Aquaculture,* **176,** 209-226.

Exadactylos, A., Geffen, A.J. Panagiota, P. & Thorpe, J.P. (2003) Population structure of Dover sole *Solea solea*; RAPD and allozyme data indicate divergence in European stocks. *Marine Ecology Progress Series,* **246,** 253-264.

FAO (2018) Food and Agriculture Organization of the United Nations, www.fao.org. (Accessed on 15/12/2018)

Fernandez, I. & Gisbert, E. (2011) The effect of vitamin A on flatfish development and skeletogenesis: a review. *Aquaculture,* **315,** 34–48.

Fernandez, I., Pimentel, M.S., Ortiz-Delgado, J.B., Hontoria, F., Sarasquete, C., Estevez, A., Zambonino-Infante, J.L. & Gisbert, E. (2009) Effect of dietary vitamin A on Senegalese sole (*Solea senegalensis*) skeletogenesis and larval quality. *Aquaculture,* **295,** 250-265.

Fernandez-Diaz, C., Yufera, M., Canavate, J.P., Moyano, F.J., Alarcon, F.J. & Diaz, M. (2001) Growth and physiological changes during metamorphosis of Senegal sole reared in the laboratory. *Journal of Fish Biology,* **58,** 1086-1097.

Ferraresso, S., Bonaldo, A., Parma, L., Cinotti, S., Massi, P., Bargelloni, L. & Gatta, P.P. (2013) Exploring the larval transcriptome of the common sole (*Solea solea* L.). *Bmc Genomics,* **14**.

Fishbase (2018) www.fishbase.org, Ed: Froese, R. & Pauly, D. (Accessed on 16/12/2018)

Fjelldal, P.G., Grotmol, S., Kryvi, H., Taranger, G.L., Hansen, T., Porter, M.J.R. & Totland, G.K. (2004) Pinealectomy induces malformation of the spine and reduces the mechanical strength of the vertebrae in Atlantic salmon, *Salmo salar*. *Journal of Pineal Research,* **36,** 132-139.

Fjelldal, P.G., Nordgarden, U., Wargelius, A., Taranger, G.L., Waagbo, R. & Olsen, R.E. (2010) Effects of vegetable feed ingredients on bone health in Atlantic salmon. *Journal of Applied Ichthyology,* **26,** 327-333.

Foekema, E.M., Deerenberg, C.M. & Murk, A.J. (2008) Prolonged ELS test with the marine flatfish sole (*Solea solea*) shows delayed toxic effects of previous exposure to PCB 126. *Aquatic Toxicology,* **90,** 197-203.

Fonds, M. (1979) Laboratory Observations on the Influence of Temperature and Salinity on Development of the Eggs and Growth of the Larvae of *Solea Solea* (Pisces). *Marine Ecology Progress Series,* **1,** 91-99.

Forberg, T., Arukwe, A. & Vadstein, O. (2011) A protocol and cultivation system for gnotobiotic Atlantic cod larvae (*Gadus morhua* L.) as a tool to study host microbe interactions. *Aquaculture,* **315,** 222-227.

Forberg, T., Vestrum, R.I., Arukwe, A. & Vadstein, O. (2012) Bacterial composition and activity determines host gene-expression responses in gnotobiotic Atlantic cod (*Gadus morhua*) larvae. *Veterinary Microbiology,* **157,** 420-427.

Fuchs, J. (1982) The Production of Juvenile Sole (*Solea Solea*) under Intensive Conditions .1. The 1st-Month of Rearing. *Aquaculture,* **26,** 321-337.

Garoia, F., Guarniero, I., Grifoni, D., Marzola, S. & Tinti, F. (2007) Comparative analysis of AFLPs and SSRs efficiency in resolving population genetic structure of Mediterranean *Solea vulgaris. Molecular Ecology,* **16,** 1377-1387.

Gavaia, P.J., Dinis, M.T. & Cancela, M.L. (2002) Osteological development and abnormalities of the vertebral column and caudal skeleton in larval and juvenile stages of hatchery-reared Senegal sole (*Solea senegalensis*). *Aquaculture,* **211,** 305-323.

Gavaia, P.J., Domingues, S., Engrola, S., Drake, P., Sarasquete, C., Dinis, M.T. & Cancela, M.L. (2009) Comparing skeletal development of wild and hatchery-reared Senegalese sole (*Solea senegalensis*, Kaup 1858): evaluation in larval and postlarval stages. *Aquaculture Research,* **40,** 1585-1593.

Goncalves, A.A. & Gagnon, G.A. (2011) Ozone Application in Recirculating Aquaculture System: An Overview. *Ozone-Science & Engineering,* **33,** 345-367.

Grotmol, S., Dahl-Paulsen, E. & Totland, G.K. (2003) Hatchability of eggs from Atlantic cod, turbot and Atlantic halibut after disinfection with ozonated seawater. *Aquaculture,* **221,** 245-254.

Guillou, A. (1978) Biologie et niveau d'exploitation des stocks de langoustine, de merlu, des baudroies et de sole dans le proche Atlantique. *Revue des travaux de l'Institut des pêches maritimes,* **42,** 155-157.

Hansen, G.H. & Olafsen, J.A. (1989) Bacterial-Colonization of Cod (*Gadus Morhua* L) and Halibut (*Hippoglossus Hippoglossus*) Eggs in Marine Aquaculture. *Applied and Environmental Microbiology,* **55,** 1435-1446.

Harboe, T., Huse, I. & Oie, G. (1994) Effects of Egg Disinfection on Yolk-Sac and 1st Feeding Stages of Halibut (*Hippoglossus Hippoglossus* L) Larvae. *Aquaculture,* **119,** 157-165.

Hirazawa, N., Hara, T., Mitsuboshi, T., Okazaki, J. & Hata, K. (1999) Iodophor disinfection of eggs of spotted halibut *Verasper variegatus* and red sea bream *Pagrus major*. *Fisheries Science,* **65,** 333-338.

Hjelm, M., Bergh, O., Riaza, A., Nielsen, J., Melchiorsen, J., Jensen, S., Duncan, H., Ahrens, P., Birkbeck, H. & Gram, L. (2004) Selection and identification of autochthonous potential probiotic bacteria from turbot larvae (*Scophthalmus maximus*) rearing units. *Systematic and Applied Microbiology,* **27,** 360-371.

Houghton, R.G., Last, J.M. & Bromley, P.J. (1985) Fecundity and Egg Size of Sole (*Solea Solea* (L)) Spawning in Captivity. *Journal Du Conseil,* **42,** 162-165.

Howell, B.R., 1973. Marine fish culture in Britain VIII. A marine rotifer, *Brachionus plicatilis* Muller, and the larvae of the mussel, *Mytilus edulis* L., as foods for larval flatfish. *Journal du Conseil / Conseil Permanent International pour l'Exploration de la Mer,* **35**, l-6.

Howell, B.R. (1997) A re-appraisal of the potential of the sole, *Solea solea* (L.), for commercial cultivation. *Aquaculture,* **155,** 355-365.

Howell, B.R. & Tzoumas, T.S. (1991) The nutritional value of *Artemia* nauplii for larval sole, *Solea solea* (L.1, with respect to their (n -31 HUFA content. In: Lavens, P., Sorgeloos, P., Jasper& E., Ollevier, F. (Eds.), Larvi '91 Fish and Crustacean Larviculture Symposium, Gent, Belgium. *European Aquaculture Society*, **Special Publication No. 15**, 63-65.

Huys, L., Dhert, P., Robles, R., Ollevier, F., Sorgeloos, P. & Swings, J. (2001) Search for beneficial bacterial strains for turbot (*Scophthalmus maximus* L.) larviculture. *Aquaculture,* **193,** 25-37.

ICES (2018) Sole (*Solea solea*) in Subarea 4 (North Sea). ICES Advice on fishing opportunities, catch, and effort. Greater North Sea Ecoregion. sol.27.4

ICES Fishmap (2018) ICES Fishmap- species factsheet *Solea solea*

Imsland, A.K., Foss, A., Conceicao, L.E.C., Dinis, M.T., Delbare, D., Schram, E., Kamstra, A., Rema, P. & White, P. (2003) A review of the culture potential of *Solea solea* and *S. senegalensis*. *Reviews in Fish Biology and Fisheries,* **13,** 379-407.

Imsland, A.K., Wergeland, T., Jonassen, T.M. & Stefansson, S.O. (2006) Does malpigmentation improve growth in juvenile turbot (*Scophthalmus maximus* Rafinesque) and halibut (*Hippoglossus hippoglossus* L.)? *Aquaculture Research,* **37,** 306-312.

Jurado, J., Villasanta-Gonzalez, A., Tapia-Paniagua, S.T., Balebona, M.C., De La Banda, I.G., Morinigo, M.A. & Prieto-Alamo, M.J. (2018) Dietary administration of the probiotic *Shewanella putrefaciens* Pdp11 promotes transcriptional changes of genes involved in growth and immunity in *Solea senegalensis* larvae. *Fish & Shellfish Immunology,* **77,** 350-363.

Kjørsvik, E., Mangorjensen, A. & Holmefjord, I. (1990) Egg Quality in Fishes. *Advances in Marine Biology,* **26,** 71-113.

Kolkovski, S. (2001) Digestive enzymes in fish larvae and juveniles - implications and applications to formulated diets. *Aquaculture,* **200,** 181-201.

Kotoulas, G., Bonhomme, F. & Borsa, P. (1995) Genetic-Structure of the Common Sole *Solea Vulgaris* at Different Geographic Scales. *Marine Biology,* **122,** 361-375.

Lagardère, F., Boulhic, M. & Burgin, T. (1993) Anomalies in the Cephalic Area of Laboratory-Reared Larvae and Juveniles of the Common Sole, *Solea Solea* - Oral Jaw Apparatus, Dermal Papillae and Pigmentation. *Environmental Biology of Fishes,* **36,** 35-46.

Lavens, P. & Sorgeloos, P. (2000) The history, present status and prospects of the availability of *Artemia* cysts for aquaculture. *Aquaculture,* **181,** 397-403.

Lewis-Mccrea, L.M. & Lall, S.P. (2010) Effects of phosphorus and vitamin C deficiency, vitamin A toxicity, and lipid peroxidation on skeletal abnormalities in Atlantic halibut (*Hippoglossus hippoglossus*). *Journal of Applied Ichthyology,* **26,** 334-343.

Li, X., Defoirdt, T., Yang, Q., Laureau, S., Bossier, P. & Dierckens, K. (2014) Host-induced increase in larval sea bass mortality in a gnotobiotic challenge test with *Vibrio anguillarum*. *Diseases of Aquatic Organisms,* **108,** 211-216.

Lobo, C., Moreno-Ventas, X., Tapia-Paniagua, S., Rodriguez, C., Morinigo, M.A. & De La Banda, I.G. (2014) Dietary probiotic supplementation (*Shewanella putrefaciens* Pdp11) modulates gut microbiota and promotes growth and condition in Senegalese sole larviculture. *Fish Physiology and Biochemistry,* **40,** 295-309.

Lund, I. (2007) The impact of dietary fatty acids in common sole larval (*Solea solea* L.) nutrition. PhD dissertation, Department of Aquaculture, Danish Technical University, Danmark.

Lund, I., Steenfeldt, S.J. & Hansen, B.W. (2007) Effect of dietary arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid on survival, growth and pigmentation in larvae of common sole (*Solea solea* L.). *Aquaculture,* **273,** 532-544.

Lund, I., Steenfeldt, S.J. & Hansen, B.W. (2010) Influence of dietary arachidonic acid combined with light intensity and tank colour on pigmentation of common sole (*Solea solea* L.) larvae. *Aquaculture,* **308,** 159-165.

Magariños, B., Romalde, J.L., López-Romalde, S., Moriñigo, M.A. & Toranzo, A.E. (2003) Pathobiological characterization of *Photobacterium damselae* subsp. *piscicida* strains isolated from cultured sole (*Solea senegalensis*). *Bulletin of the European Association of Fish Pathologists*, **23**.

Mahious, A.S., Gatesoupe, F.J., Hervi, M., Metailler, R. & Ollevier, F. (2006) Effect of dietary inulin and oligosaccharides as prebiotics for weaning turbot, *Psetta maxima* (Linnaeus, C. 1758). *Aquaculture International,* **14,** 219-229.

Makridis, P., Bergh, Ø., Skjermo, J. & Vadstein, O. (2001) Addition of bacteria bioencapsulated in *Artemia* metanauplii to a rearing system for halibut larvae. *Aquaculture International,* **9,** 225-235.

Makridis, P., Martins, S., Reis, J. & Dinis, M.T. (2008) Use of probiotic bacteria in the rearing of Senegalese sole (*Solea senegalensis*) larvae. *Aquaculture Research,* **39,** 627-634.

Manfrin, A., Doimi, M., Antonetti, P,. Delgado Montero, M.L., Qualtieri, K., Rampazzo, E., Vascellari, M., Mutinelli, F., Melchiotti, E., Bozza, M.A., Selli, L. & Bovo, G. (2003) Primo isolamento di *Vibrio anguillarum* sierotipo O 1 da sogliola (*Solea solea*, L.) in Itali/ First isolation of *Vibrio anguillarum* O1 serotype from sole (*Solea solea*) in Italy. *Bollettino Societa Italiana di Patologia Ittica* **37,** 12-17.

Marques, A., Ollevier, F., Verstraete, W., Sorgeloos, P. & Bossier, P. (2005) Gnotobiotically grown aquatic animals: opportunities to investigate host-microbe interactions. *Journal of applied Microbiology*, **100**, 903-918.

Martin, I., Rasines, I., Gomez, M., Rodriguez, C., Martinez, P. & Chereguini, O. (2014) Evolution of egg production and parental contribution in Senegalese sole, *Solea senegalensis*, during four consecutive spawning seasons. *Aquaculture,* **424,** 45-52.

McEvoy, L.A., Naess, T., Bell, J.G. & Lie, O. (1998) Lipid and fatty acid composition of normal and malpigmented Atlantic halibut (*Hippoglossus hippoglossus*) fed enriched *Artemia*: a comparison with fry fed wild copepods. *Aquaculture,* **163,** 237-250.

McGurk, M.D. (1984) Effects of Delayed Feeding and Temperature on the Age of Irreversible Starvation and on the Rates of Growth and Mortality of Pacific Herring Larvae. *Marine Biology,* **84,** 13-26.

McKenzie, D.J., Lund, I. & Pedersen, P.B. (2008) Essential fatty acids influence metabolic rate and tolerance of hypoxia in Dover sole (*Solea solea*) larvae and juveniles. *Marine Biology,* **154,** 1041-1051.

Merrifield, D.L., Dimitroglou, A., Foey, A., Davies, S.J., Baker, R.T.M., Bogwald, J., Castex, M. & Ringø, E. (2010) The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture,* **302,** 1-18.

Meyer, F.P. (1991) Aquaculture Disease and Health Management. *Journal of Animal Science,* **69,** 4201-4208.

Morais, S. , Aragão, C. , Cabrita, E. , Conceição, L. E., Constenla, M. , Costas, B. , Dias, J. , Duncan, N. , Engrola, S. , Estevez, A. , Gisbert, E. , Mañanós, E. , Valente, L. M., Yúfera, M. & Dinis, M. T. (2016) New developments and biological insights into the farming of *Solea senegalensis* reinforcing its aquaculture potential. *Reviews in Aquaculture*, **8,** 227-263.

Morrison, C., Bird, C., O'neil, D., Leggiadro, C., Martin-Robichaud, D., Rommens, M. & Waiwood, K. (1999) Structure of the egg envelope of the haddock, *Melanogrammus aeglefinus*, and effects of microbial colonization during incubation. *Canadian Journal of Zoology-Revue Canadienne De Zoologie,* **77,** 890-901.

Mourente G. & Vásquez R. (1996) Changes in the content of total lipid, lipid classes and their fatty acids of developing eggs and unfed larvae of the senegal sole, *Solea senegalensis* Kaup. *Fish Physiology and Biochemistry*, **15**, 221-235.

Munk, P., Nielsen, J.G. (2005) *Eggs and larvae of North Sea fishes*. Biofolia, Frederiksberg, Danmark, 1-215.

Munro, P.D., Barbour, A. & Birkbeck, T.H. (1995) Comparison of the Growth and Survival of Larval Turbot in the Absence of Culturable Bacteria with Those in the Presence of *Vibrio Anguillarum*, *Vibrio Alginolyticus*, or a Marine *Aeromonas* Sp. *Applied and Environmental Microbiology,* **61,** 4425-4428.

Muroga, K., Yasunobu, H., Okada, N. & Masumura, K. (1990) Bacterial Enteritis of Cultured Flounder *Paralichthys Olivaceus* Larvae. *Diseases of Aquatic Organisms,* **9,** 121-125.

Murray, H.M., Lall, S.P., Rajaselvam, R., Boutilier, L.A., Blanchard, B., Flight, R.M., Colombo, S., Mohindra, V. & Douglas, S.E. (2010) A nutrigenomic analysis of intestinal response to partial soybean meal replacement in diets for juvenile Atlantic halibut, *Hippoglossus hippoglossus*, L. *Aquaculture,* **298,** 282-293.

Nayak, S.K. (2010) Probiotics and immunity: A fish perspective. *Fish & Shellfish Immunology,* **29,** 2-14.

Nichols, J. H. (1976) Soleidae of the eastern-north Atlantic. Fiches d'Identification du Zooplancton, 1-10.

OECD (2013) Test No. 236: Fish embryo acute toxicity (FET) test, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.

Ohs, C., Cassiano, E. J. & Rhodes, A. (2012) Choosing an Appropriate Live Feed for Larviculture of Marine Fish. Fisheries and Aquatic Sciences Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida. FA167.

Olafsen, J.A. (2001) Interactions between fish larvae and bacteria in marine aquaculture. *Aquaculture,* **200,** 223-247.

Olsen, A.I., Olsen, Y., Attramadal, Y., Christie, K., Birkbeck, T.H., Skjermo, J. & Vadstein, O. (2000) Effects of short term feeding of microalgae on the bacterial flora associated with juvenile *Artemia franciscana*. *Aquaculture,* **190,** 11-25.

Olsson, J.C., Joborn, A., Westerdahl, A., Blomberg, L., Kjelleberg, S. & Conway, P.L. (1998) Survival, persistence and proliferation of *Vibrio anguillarum* in juvenile turbot, *Scophthalmus maximus* (L.), intestine and faeces. *Journal of Fish Diseases,* **21,** 1-9.

Padros, F., Villalta, M., Gisbert, E. & Estevez, A. (2011) Morphological and histological study of larval development of the Senegal sole *Solea senegalensis*: an integrative study. *Journal of Fish Biology,* **79,** 3-32.

Palazzi, R., Richard, J., Bozzato, G. & Zanella, L. (2006) Larval and juvenile rearing of common sole (*Solea solea* L.) in the Northern Adriatic (Italy). *Aquaculture,* **255,** 495-506.

Palermo, F.A., Mosconi, G., Avella, M.A., Carnevali, O., Verdenelli, M.C., Cecchini, C. & Polzonetti-Magni, A.M. (2011) Modulation of cortisol levels, endocannabinoid receptor 1A, proopiomelanocortin and thyroid hormone receptor alpha mRNA expressions by probiotics during sole (*Solea solea*) larval development. *General and Comparative Endocrinology,* **171,** 293-300.

Paolini, A., Magi, G.E., Gennari, L., Egidi, C., Torresi, M., Vallerani, M., Berti, M., Pavone, A. & Giorgetti, G. (2010). Severe mortality in common sole (*Solea solea*) at post-weaning stage following environmental stress. *Bulletin of the European Association of Fish Pathologists,* **30**, 160-169.

Parma, L., Bonaldo, A., Massi, P., Yufera, M., Martinez-Rodriguez, G. & Gatta, P.P. (2013) Different early weaning protocols in common sole (*Solea solea* L.) larvae: Implications on the performances and molecular ontogeny of digestive enzyme precursors. *Aquaculture,* **414,** 26-35.

Peeler, E.J., Oidtmann, B.C., Midtlyng, P.J., Miossec, L. & Gozlan, R.E. (2011) Non-native aquatic animals introductions have driven disease emergence in Europe. *Biological Invasions,* **13,** 1291-1303.

Pham, L.N., Kanther, M., Semova, I. & Rawls, J.F. (2008) Methods for generating and colonizing gnotobiotic zebrafish. *Nature Protocols,* **3,** 1862-1875.

Piccinetti, C.C., Ricci, L.A., Tokle, N., Radaelli, G., Pascoli, F., Cossignani, L., Palermo, F., Mosconi, G., Nozzi, V., Raccanello, F. & Olivotto, I. (2012) Malnutrition may affect common sole (*Solea solea* L.) growth, pigmentation and stress response: Molecular, biochemical and histological implications. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology,* **161,** 361-371.

Planas, M., Perez-Lorenzo, M., Hjelm, M., Gram, L., Fiksdal, I.U., Bergh, O. & Pintado, J. (2006) Probiotic effect in vivo of *Roseobacter strain* 27-4 against *Vibrio (Listonella) anguillarum* infections in turbot (*Scophthalmus maximus* L.) larvae. *Aquaculture,* **255,** 323-333.

Rasines, I., Gómez, M., Martín, I., Rodríguez, C., Mañanós, E. & Chereguini, O. (2013) Artificial fertilisation of cultured Senegalese sole (*Solea senegalensis*): effects of the time of day of hormonal treatment on inducing ovulation. *Aquaculture*, **392–395**, 94-97.

Rawls, J.F., Samuel, B.S. & Gordon, J.I. (2004) Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proceedings of the National Academy of Sciences of the United States of America,* **101,** 4596-4601.

Reitan, K.I., Rainuzzo, J.R., Olsen, Y. (1994). Influence of live feed on growth, survival and pigmentation of turbot larvae. *Aquaculture International*, **2**, 33–48.

Ribeiro, L., Zambonino-Infante, J.L., Cahu, C. & Dinis, M.T. (1999) Development of digestive enzymes in larvae of *Solea senegalensis*, Kaup 1858. *Aquaculture,* **179,** 465-473.

Richard, P., Bergeron, J.P., Boulhic, M., Galois, R. & Personleruyet, J. (1991) Effect of Starvation on RNA, DNA and Protein-Content of Laboratory-Reared Larvae and Juveniles of *Solea Solea*. *Marine Ecology Progress Series,* **72,** 69-77.

Richard, N., Fernandez, I., Wulff, T., Hamre, K., Cancela, L., Conceicao, L.E.C. & Gavaia, P.J. (2014) Dietary supplementation with vitamin K affects transcriptome and proteome of Senegalese sole, improving larval performance and quality. *Marine Biotechnology*, **16**, 522–537.

Rijnsdorp, A.D., Vanbeek, F.A., Flatman, S., Millner, R.M., Riley, J.D., Giret, M. & Declerck, R. (1992) Recruitment of Sole Stocks, *Solea Solea* (L), in the Northeast Atlantic (Netherlands Journal of Sea Research, Vol 29, Pg 173, 1992). *Netherlands Journal of Sea Research,* **29,** U402-U402.

Rijnsdorp, A.D. & Vingerhoed, B. (1994) The Ecological Significance of Geographical and Seasonal Differences in Egg Size in Sole *Solea Solea* (L). *Netherlands Journal of Sea Research,* **32,** 255-270.

Ringø, E., Dimitroglou, A., Hoseinifar, S. H. & Davies, S.J. (2014a) Prebiotics in finfish: an update. In: *Aquaculture Nutrition: GutHealth, Probiotics and Prebiotics*, First Edition. Ed: Merrifield, D. & Ringø, E, John Wiley & Sons, Hoboken, New Jersey, United States.

Ringø, E., Olsen, R.E., Gifstad, T.O., Dalmo, R.A., Amlund, H., Hemre, G.I. & Bakke, A.M. (2010) Prebiotics in aquaculture: a review. *Aquaculture Nutrition,* **16,** 117-136.

Ringø, E., Olsen, R.E., Jensen, I., Romero, J. & Lauzon, H.L. (2014b) Application of vaccines and dietary supplements in aquaculture: possibilities and challenges. *Reviews in Fish Biology and Fisheries,* **24,** 1005-1032.

Ringø, E., Olsen, R.E., Vecino J.L.G., Wadsworth, S. & Song, S.K. (2012) Use of immunostimulants and nucleotides in aquaculture: a review. *Journal of Marine Science: Research and Development*, **2**.

Ringø, E. & Vadstein, O. (1998) Colonization of *Vibrio pelagius* and *Aeromonas caviae* in early developing turbot (*Scophthalmus maximus* L.) larvae. *Journal of Applied Microbiology,* **84,** 227-233.

Rolland, J.L., Bonhomme, F., Lagardère, F., Hassan, M. & Guinand, B. (2007) Population structure of the common sole (*Solea solea*) in the Northeastern Atlantic and the Mediterranean Sea: revisiting the divide with EPIC markers. *Marine Biology*, **151**, 1-17.

Romero, J., Feijoo, C.G. & Navarrete, P. (2012) Antibiotics in Aquaculture – Use, Abuse and Alternatives. In: Carvalho ED, David GS, Silva RJ (eds) *Health and Environment in Aquaculture*. InTech, Rijeka, 159-198.

Rønneseth, A., Castillo, D., D'alvise, P., Tonnesen, O., Haugland, G., Grotkjaer, T., Engell-Sorensen, K., Norremark, L., Bergh, O., Wergeland, H.I. & Gram, L. (2017) Comparative assessment of *Vibrio* virulence in marine fish larvae. *Journal of Fish Diseases,* **40,** 1373-1385.

Rosenlund, G., Stoss, J. & Talbot, C. (1997) Co-feeding marine fish larvae with inert and live diets. *Aquaculture,* **155,** 183-191.

Saele, O., Solbakken, J.S., Watanabe, K., Hamre, K., Power, D. & Pittman, K. (2004) Staging of Atlantic halibut (*Hippoglossus hippoglossus* L.) from first feeding through metamorphosis, including cranial ossification independent of eye migration. *Aquaculture,* **239,** 445-465.

Salvesen, I., Jørgensen, L. & Vadstein, O. (1991) Evaluation of four chemicals for surface-disinfection of marine fish eggs. In: *LARVI '91 - Fish & Crustacean larviculture symposium* (ed. by P. Lavens, P. Sorgeloos, E. Jaspers & F. Ollevier). European Aquaculture Society, special publication, Ghent, Belgium.

Salvesen, I., Oie, G. & Vadstein, O. (1997) Surface disinfection of Atlantic halibut and turbot eggs with glutaraldehyde: Evaluation of concentrations and contact times. *Aquaculture International,* **5,** 249-258.

Schaeck, M., De Swaef, E., Van Den Broeck, W., Van Nevel, S., Boon, N., De Geyter, N., Morent, R., Demeestere, K., Duchateau, L., Coulombet, C., Haesebrouck, F. & Decostere, A. (2016) Germ-free sea

bass *Dicentrarchus labrax* larval model: a valuable tool in the study of host-microbe interactions. *Diseases of Aquatic Organisms,* **117,** 177-185.

Schreiber, A.M. (2006) Asymmetric craniofacial remodeling and lateralized behavior in larval flatfish. *Journal of Experimental Biology,* **209,** 610-621.

Shields, R.J., Bell, J.G., Luizi, F.S., Gara, B., Bromage, N.R. & Sargent, J.R. (1999) Natural copepods are superior to enriched *Artemia* nauplii as feed for halibut larvae (*Hippoglossus hippoglossus*) in terms of survival, pigmentation and retinal morphology: Relation to dietary essential fatty acids. *Journal of Nutrition,* **129,** 1186-1194.

Silva, Y.J., Costa, L., Pereira, C., Mateus, C., Cunha, A., Calado, R., Gomes, N.C.M., Pardo, M.A., Hernandez, I. & Almeida, A. (2014) Phage Therapy as an Approach to Prevent *Vibrio anguillarum* Infections in Fish Larvae Production. *Plos One,* **9**.

Situmorang, M.L., Dierckens, K., Mlingi, F.T., Van Delsen, B. & Bossier, P. (2014) Development of a bacterial challenge test for gnotobiotic Nile tilapia *Oreochromis niloticus* larvae. *Diseases of Aquatic Organisms,* **109,** 23-33.

Skjermo, J. & Bergh, O. (2004) High-M alginate immunostimulation of Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae using Artemia for delivery, increases resistance against vibriosis. *Aquaculture,* **238,** 107-113.

Skjermo, J., Defoort, T., Dehasque, M., Espevik, T., Olsen, Y., Skjakbraek, G., Sorgeloos, P. & Vadstein, O. (1995) Immunostimulation of Juvenile Turbot (*Scophthalmus Maximus* L) Using an Alginate with High Mannuronic Acid Content Administered Via the Live Food Organism Artemia. *Fish & Shellfish Immunology,* **5,** 531-534.

Skjermo, J. & Vadstein, O. (1999) Techniques for microbial control in the intensive rearing of marine larvae. *Aquaculture,* **177,** 333-343.

Sorensen, S.R., Skov, P.V., Lauesen, P., Tomkiewicz, J., Bossier, P. & De Schryver, P. (2014) Microbial interference and potential control in culture of European eel (*Anguilla anguilla*) embryos and larvae. *Aquaculture,* **426,** 1-8.

Strand, H.K. & Dalmo, R.A. (1997) Absorption of immunomodulating beta(1,3)-glucan in yolk sac larvae of Atlantic halibut, *Hippoglossus hippoglossus* (L). *Journal of Fish Diseases,* **20,** 41-49.

Stuart, K.R., Keller, M. & Drawbridge, M. (2010) Efficacy of formalin and povidone-iodine disinfection techniques on the eggs of three marine finfish species. *Aquaculture Research,* **41,** e838-e843.

Sugita, H., Hirose, Y., Matsuo, N. & Deguchi, Y. (1998) Production of the antibacterial substance by *Bacillus* sp. strain NM 12, an intestinal bacterium of Japanese coastal fish. *Aquaculture,* **165,** 269-280.

Taoka, Y., Maeda, H., Jo, J.Y., Jeon, M.J., Bai, S.C., Lee, W.J., Yuge, K. & Koshio, S. (2006) Growth, stress tolerance and non-specific immune response of Japanese flounder *Paralichthys olivaceus* to probiotics in a closed recirculating system. *Fisheries Science,* **72,** 310-321.

Tapia-Paniagua, S.T., Vidal, S., Lobo, C., Prieto-Alamo, M.J., Jurado, J., Cordero, H., Cerezuela, R., De La Banda, I.G., Esteban, M.A., Balebona, M.C. & Morinigo, M.A. (2014) The treatment with the probiotic *Shewanella putrefaciens* Pdp11 of specimens of *Solea senegalensis* exposed to high stocking densities to enhance their resistance to disease. *Fish & Shellfish Immunology,* **41,** 209-221.

Toranzo, A.E. (2004) Report about fish bacterial diseases**.** In: Alvarez-Pellitero, P. (ed.), Barja, J.L. (ed.), Basurco, B. (ed.), Berthe, F. (ed.) & Toran, zo A.E. (ed.). *Mediterranean aquaculture diagnostic laboratories.* (Options Méditerranéennes: Série B. Etudes et Recherches; n.49) Zaragoza: CIHEAM, Spain, 49 -89.

Toranzo, A.E., Magarinos, B. & Romalde, J.L. (2005) A review of the main bacterial fish diseases in mariculture systems. *Aquaculture,* **246,** 37-61.

Touraki, M., Niopas, I. & Karagiannis, V. (2012) Treatment of vibriosis in European sea bass larvae, *Dicentrarchus labrax* L., with oxolinic acid administered by bath or through medicated nauplii of *Artemia franciscana* (Kellogg): efficacy and residual kinetics. *Journal of Fish Diseases,* **35,** 513-522.

Tveiten, H. & Tromsø, N.M. (2009) 10.1 Early development, maternal effects and endocrinology. In: The fish larva: a transitional life form, the foundation for aquaculture and fisheries. The Research Council of Norway. 41-52.

UN (2014) United Nations, Department of Economics, Concise Report on the World Population Situation in 2014. ST/ESA/SER.A/354, United Nations Publications.

Vadstein, O. (1997) The use of immunostimulation in marine larviculture: possibilities and challenges. *Aquaculture,* **155,** 401-417.

Vadstein, O., Øie, G., Olsen, Y., Salvesen, I., Skjermo, J. & Skjak-Bræk, G. (1993) A strategy to obtain microbial control during larval development of marine fish. In: Reinertsen, H., Dahle, L.A., Jørgensen, L., Tvinnereim, K. (Eds.), *Fish Farming Technology*. Proceedings of the First International Conference on Fish Farming Technology, 9 –12 August 1993, Trondheim, Norway. A.A. Balkema, Rotterdam, 69–75.

Verner-Jeffreys, D.W., Shields, R.J. & Birkbeck, T.H. (2003) Bacterial influences on Atlantic halibut *Hippoglossus hippoglossus* yolk-sac larval survival and start-feed response. *Diseases of Aquatic Organisms,* **56,** 105-113.

Villalta, M., Estevez, A. & Bransden, M.P. (2005) Arachidonic acid enriched live prey induces albinism in Senegal sole (*Solea senegalensis*) larvae. *Aquaculture,* **245,** 193-209.

Vine, N.G., Leukes, W.D. & Kaiser, H. (2006) Probiotics in marine larviculture. *Fems Microbiology Reviews,* **30,** 404-427.

Wagemans, F. & Vandewalle, P. (2001) Development of the bony skull in common sole: brief survey of morpho-functional aspects of ossification sequence. *Journal of Fish Biology,* **59,** 1350-1369.

Wang, Y.N., Zhang, Y., Li, X., Sun, M.Z., Wei, Z., Wang, Y., Gao, A.A., Chen, D.Y., Zhao, X. & Feng, X.Z. (2015) Exploring the Effects of Different Types of Surfactants on Zebrafish Embryos and Larvae. *Scientific Reports,* **5**.

Witten, P.E., Gil-Martens, L., Hall, B.K., Huysseune, A. & Obach, A. (2005) Compressed vertebrae in Atlantic salmon *Salmo salar*: evidence for metaplastic chondrogenesis as a skeletogenic response late in ontogeny. *Diseases of Aquatic Organisms,* **64,** 237-246.

Ye, J.D., Wang, K., Li, F.D. & Sun, Y.Z. (2011) Single or combined effects of fructo- and mannan oligosaccharide supplements and *Bacillus clausii* on the growth, feed utilization, body composition, digestive enzyme activity, innate immune response and lipid metabolism of the Japanese flounder *Paralichthys olivaceus*. *Aquaculture Nutrition,* **17,** E902-E911.

Yufera, M. (2018) Emerging Issues in Fish Larvae Research. Springer International Publishing, Cham, Switzersland.

Zorrilla, I., Balebona, M.C., Moriñigo, M.A., Sarasquete, C. & Borrego, J.J. (1999) Isolation and characterization of the causative agent of pasteurellosis, *Photobacterium damsela* ssp. *piscicida*, from sole *Solea senegalensis* (Kaup). *Journal of Fish Diseases*, **22**, 167-171.

Chapter 2 AIMS OF THE STUDY

Aquaculture is the fastest growing source of animal protein in the world and based on the increasing human population, a continuously rising demand for fish through aquaculture is to be expected in the future. In addition, the capture fisheries production is stagnant and consequently, an intensification and diversification of the aquaculture sector imposes itself. In this regard, Dover Sole is a promising aquaculture candidate as the flesh is highly appreciated in culinary circles and has a high market value. Dover sole aquaculture is hampered by the limited knowledge on the basic biology of the species, resulting amongst others in high mortality rates during the larval phase. Furthermore, disease outbreaks are increasingly recognized as significantly restricting aquaculture production. Due to the increasing awareness of the human and animal health risks associated with the extensive use of antibiotics, there is a strong emphasis on alternative preventive and control measures to combat disease. In this respect, strict hygiene measures are implemented in aquaculture facilities including routine disinfection of fish eggs. However, at the initiation of the present PhD research, no egg disinfection protocols were available for Dover sole. Likewise, multiple environmentally friendly prophylactic disease treatments including the use of immunostimulants such as probiotics and prebiotics were pinpointed for various marine fish larval species, but not for Dover sole larvae. The above mentioned hindrances in Dover sole larviculture and in particular the very limited knowledge on this species, stress the need for increased research efforts concerning its larviculture.

In view of the above, the overall aim of this dissertation is to contribute to more sustainable Dover sole larviculture by developing several innovative research tools deployable to tackling some key issues hampering its development. An additional target is to evaluate several probiotic and prebiotic candidates to increase Dover sole larval health and control the bacterial disease vibriosis.

In order to achieve this, the following specific objectives were defined:

A. to identify possible disinfection methods that may be implemented for Dover sole eggs by reviewing the current egg disinfection protocols (**chapter 3**);

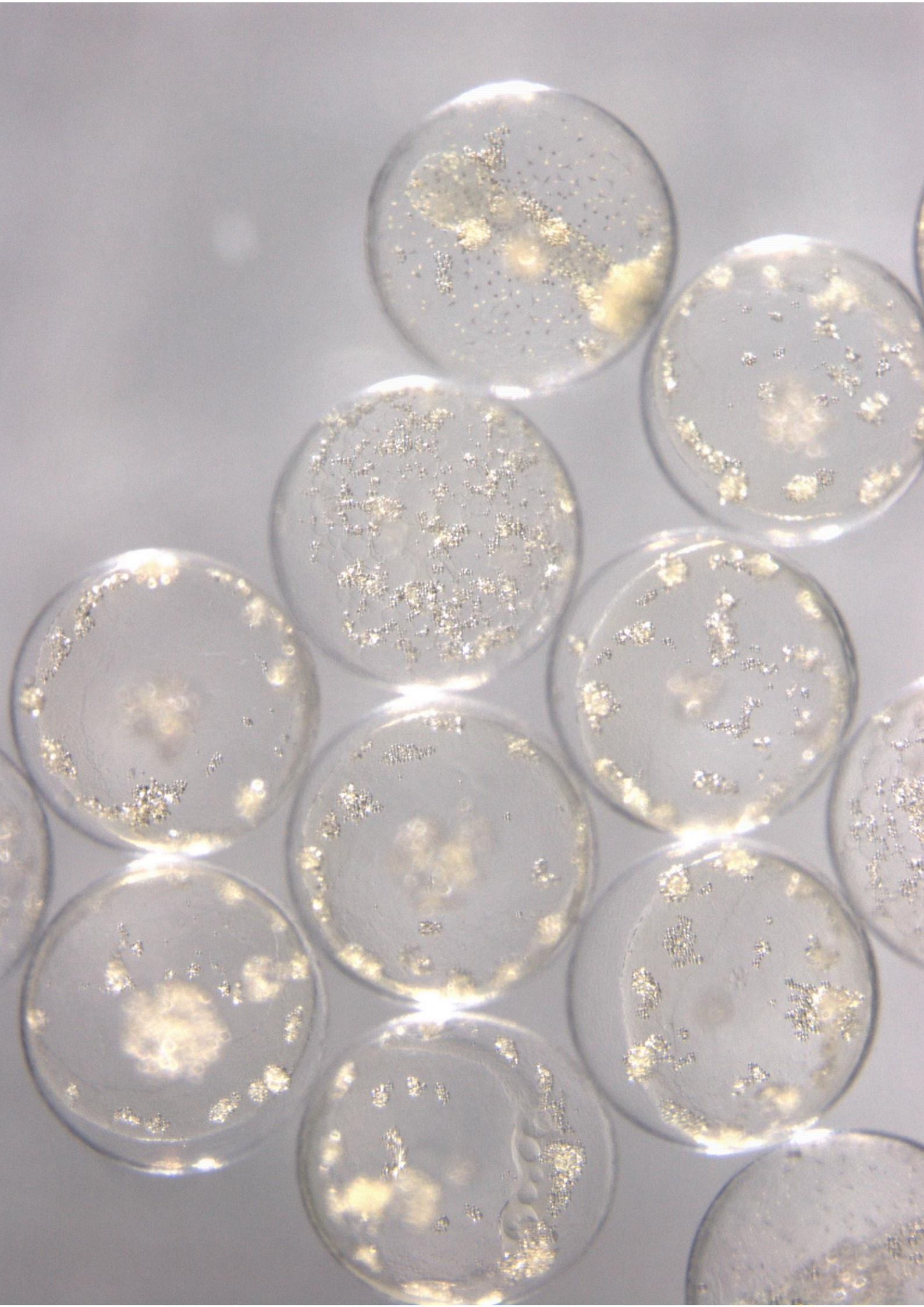
B. to determine the ultrastructural morphology of the Dover sole egg envelope from fertilization until hatching as a means to predict the influence of external factors on the Dover sole embryos. In order to achieve this, an adequate fixation and embedding protocol for scanning and transmission electron microscopy was pinpointed (**chapter 4**);

C. to develop a state-of-the-art experimental housing system for Dover sole larvae in 24-well plates, including adequate egg disinfection and examining the feasibility of developing a gnotobiotic Dover sole model (**chapter 5**);

D. to establish an experimental infection model for vibriosis in Dover sole larvae in order to enable the investigation of its pathogenesis and engender the possibility to evaluate potential prevention and treatment measures (**chapter 6**);

E. to investigate a possibly positive health impact following supplementation of a selection of probiotic strains on Dover sole larvae (**chapter 6**);

F. to assess the impact of two potentially prebiotic components, mannanoligosaccharide and alginic acid, on larval growth and survival as well as protection following *V. anguillarum* challenge in Dover sole larvae using the above pinpointed housing system and infection model (**chapter 7**).



Chapter 3 DISINFECTION OF TELEOST EGGS: A REVIEW

Based on: De Swaef, E., Van de Broeck, W., Dierckens, K. & Decostere, A. (2016) Disinfection of teleost eggs: a review. *Reviews in Aquaculture*, **8**, 321-341.

3.1 ABSTRACT

In aquaculture hatcheries, egg disinfection is commonly employed as a mortality mitigation and disease management tool. In addition, disinfection protocols are utilized in research facilities, as a means to create axenic and gnotobiotic larval models. For the latter, a complete sterilization of the eggs is warranted. Multiple research groups have tested various disinfectants in fish eggs adopting different protocols and a range of parameters to evaluate their efficacy and safety. However, there is a clear lack of a critical review listing these different studies and stressing the advantages and shortcomings of the protocols and adopted disinfectants. This review provides a commented survey of the findings/experiments on the most frequently used and upcoming disinfecting products, the protocols in which they are included and their purposes with emphasis on decreasing bacterial and/or fungal load, underscoring the benefits as well as the limitations of each product. Supplemented with a critical note, this review assists both aquaculturists and researchers in making an informed choice. Moreover, it will help stakeholders to identify the gaps in knowledge where future research on egg disinfection is needed.

3.2 INTRODUCTION

Aquaculture is the world's fastest growing source of animal protein, providing more than one third of all fish consumed globally (FAO, 2012). Because of the increasing importance of the aquaculture sector during the last decades, a constant intensification is conspicuous, to which phenomenon also the larval phase of the production cycle is subjected. However, the latter is affected by high and unpredictable mortality, causing large economic losses. These mortalities may be triggered by a wide range of causes, one of these being high bacterial densities in the rearing tanks. These may result in overgrowth of fish eggs (Hansen & Olafsen, 1989; Hansen & Olafsen, 1999; Morrison et al., 1999) and lead to a drastic reduction of the oxygen exchange, causing asphyxiation of the fish embryo (Brown et al., 2005). Besides, infection with a pathogenic bacterial agent can negatively affect survival and development during the embryonic and larval phases of the fish (Colwell & Grimes, 1984; Munro et al., 1995; Sørensen et al., 2014). In addition, fish eggs may harbor pathogenic microorganisms and hence are considered as a major route for disease transmission (Bergh et al., 1990; Brown et al., 1997; Ekman et al., 1999; Peeler et al., 2011).

In view of the above, egg disinfection can be employed as a mortality mitigation and disease management tool in hatcheries. However, disinfecting may disturb the balance of microbial communities in the rearing water, allowing opportunistic bacteria to prosper (Salvesen et al. 2000; Blancheton et al., 2013). Indeed, following disinfection, only few bacteria can profit from a large amount of nutrition and consequently, opportunistic bacteria with high growth rates may proliferate (Andrews & Harris, 1986). Disinfectants can further be adopted in research facilities, as a means to create axenic and gnotobiotic larval models (Munro et al., 1995; Verner-Jeffreys et al., 2003; Dierckens et al., 2009; Forberg et al., 2011; Situmorang et al., 2014; Schaeck et al., 2016a). For the latter, not only a decrease in bacterial load (disinfection) is warranted, but complete sterility of the egg surface needs to be achieved in order to obtain axenic larvae (Dierckens et al., 2009; Forberg et al. 2011). Fungal infections such as saprolegniasis can cause large scale problems in fish farms (Piper et al., 1982; Small & Chatakondi, 2006). Fungal spores are ubiquitous in hatchery water supplies and are able to elicit large fungal outbreaks depending on the incubation time of the egg, the water temperature or organic load. To control these outbreaks, increasing water flow rates is an option, although caution is needed to avoid damaging the eggs (Rach et al., 1995). Also handpicking of dead eggs can reduce fungal contamination but this treatment is highly labour-intensive (Barnes et al., 2001). Therefore, chemical disinfection is often used to restrict fungal overload.

Multiple research groups have tested various disinfectants in fish eggs adopting different protocols and a range of parameters to evaluate their efficacy and safety. In what follows, a survey is given of the findings/experiments on the most frequently used and upcoming disinfecting products, the protocols in which they are included and their purposes, underscoring the benefits as well as the

limitations of each product. This summary aims to help both aquaculturists and researchers to consider all aspects before implementing a specific disinfection protocol. Moreover, it will assist the scientific community in identifying the gaps in knowledge where future studies on egg disinfection are needed.

3.3 EVALUATION CRITERIA FOR A SUCCESSFUL DISINFECTION

The various parameters that are used to evaluate the disinfectants largely depend on the predefined objectives of the users. In the vast majority of cases, the prevailing interest is in the ability of the treatment to reduce the bacterial or fungal load without reducing the hatchability of the eggs. Only a minority of the researchers aims to obtain fully sterile eggs and fish larvae. The latter can be the first step in developing an axenic or gnotobiotic fish larval model, a very useful research tool e.g. to unravel the mode of action of probiotic bacteria or to pinpoint the interaction of a pathogen with its target host tissue, without interference of unknown microbiota. As exemplified further, in some cases only the tolerance of a fish species to a specific disinfectant was investigated, without looking at the actual disinfection potential of the product. For ease of understanding and to fully grasp the chapter where the various disinfectants are elaborated on, an overview is given of the parameters that were adopted for assessing their efficacy and safety.

3.3.1 Bacterial and fungal load

As stated above, only a minority of the researchers had the objective to obtain fully sterilized eggs. Most studies attempted to only reduce the bacterial and fungal load using a variety of different techniques.

3.3.1.1 Microscopy

To identify the presence of fungi on fish eggs and to evaluate the rate of fungal growth, light microscopy was used (Pavlov & Moksness, 1993; Khodabandeh & Abtahi, 2006; Straus et al., 2012a; 2016). Different authors indicated that the presence of fungi on eggs was visually documented, but whether this happened by merely observing the eggs with the naked eye (Aller-Gancedo & Fregeneda-Grandes, 2007; El-Gawad et al., 2016) or examining these microscopically, is not mentioned (Marking et al., 1994; Schreier et al., 1996; Rach et al., 1998; Forneris et al.,2003; Small, 2009). Two studies determined bacterial load based on quantifying the egg surface coverage with scanning electron microscopy (Barnes et al., 2005; Sorensen et al., 2014).

3.3.1.2 Culture media

As already reviewed in Marques et al. (2006), using bacteriological culture media alone may not give an accurate representation of the bacterial load of the sample, as a large amount of bacteria can not be cultured rendering a truthful enumeration by this technique impossible. Although the use of culture media may give an estimation of a possible reduction in bacterial load, caution is needed when claiming

sterility of fish eggs based on this technique. The culture media that were adopted can be agar based media and broths.

3.3.1.2.1 Agar based media

Throughout the studies, many different agar types were used singly or in combination. Targeting specific pathogens may oblige the researchers to use a specific selective culture medium whereas the overall bacterial load can be best detected with general purpose media. Most researchers adopted one or more general purpose media, although on occasion additionally including a selective medium as illustrated in Table 3.1. Incubation time and temperature vary greatly in between studies. Although one would expect that the incubation temperature coincides with the temperature at which the eggs are held, this is not always the case (Table 3.1). Most commonly a subsample of eggs (e.g. Peck et al., 2004; Treasurer et al., 2005) and/or water sample of the rearing containers (Escaffre et al., 2001; Forberg et al., 2011) is plated out. Another technique is to homogenise a subsample of eggs, followed by inoculating the resulting homogenate whether or not diluted (e.g. Dierckens et al., 2009; Wagner et al., 2012b) or mixing the homogenate with preheated fluid agar, before pouring into petri dishes (Ben-Atia et al., 2007; Katharios et al., 2007). Shaw (1957) tested sterility by plating dead fry instead of eggs.

3.3.1.2.2 Broths

Broths were regularly used to quantify the bacterial load or verify the sterility of the disinfected eggs. Most frequently used culture broths are Tryptic Soy Broth (TSB), Brain Heart Infusion broth (BHIB) or Marine Broth (MB), supplemented with NaCl when the presence of halophilous bacteria was tested for (Table 3.1). As for agar plates, incubation times and temperatures of culture broths vary greatly, impeding comparisons in between different protocols. Inoculation of the broth was performed with a subsample of eggs, egg homogenate or a water sample.

3.3.1.3 Cell staining techniques

Cell staining techniques are rarely used in disinfection studies although they are not culture dependent and therefore quicker.

3.3.1.3.1 DAPI

4',6-diamidino-2-fenylindool (DAPI) is a fluorescent stain that binds to DNA and can be used to demonstrate the presence of bacterial cells. With this technique also non-culturable bacteria can be detected. However, no difference between life and dead bacterial cells can be observed (Johnson, 2010). Only one study used this technique to verify the absence of bacterial cells in culture water of disinfected fish eggs (Verner-Jeffreys et al., 2003).

3.3.1.3.2 Flow cytometry

Flow cytometry is a laser based technique used to detect stained bacterial cells, but interpretation of the acquired data is difficult and the background signal needs to be distinguished from cell counts (Mohr et al., 2006; Karo et al., 2008). Forberg et al. (2011) and Schaeck et al. (2016a) used this non-culture dependent technique to confirm the sterility of disinfected cod (*Gadus morhua*) or seabass (*Dicentrarchus labrax* L.) eggs, respectively. SYBR green, whether (Schaeck et al., 2016a) or not (Forberg et al., 2011) combined with propidium iodide, was used to stain DNA particles, but also this staining technique makes no distinction between live or dead cells (Luna et al., 2002). The background signal was determined by including filtered autoclaved seawater.

3.3.1.4 Detection of bacterial DNA

To avoid the bias of culture dependent techniques, one study used DNA extraction and amplification of 16S rRNA to verify the absence of bacteria in the culture water of disinfected fish eggs (Dierckens et al., 2009). Although in theory, PCR-based techniques can detect one single bacterial cell, in practice, many different factors may intermingle with this technique (Hiney & Smith, 1998). Indeed, in the context of sterility tests for disinfected fish eggs, it is important to address the problem of possible false negative results. These can be caused by unsuitable products or protocols which increase the lower detection limit. In addition, the presence of too much non-target DNA may inhibit the assay (reviewed in Wilson (1997)). Next to false negative, also false positive results may be observed due to the multiplication of DNA of dead bacterial cells (Satokari et al., 1998) or binding of bacterial primers to eukaryote DNA (Dierckens et al., 2009). To remediate the latter, Bakke et al. (2011) present a PCR strategy to obtain pure bacterial rDNA amplicons from samples predominated by eukaryotic DNA. To determine the bacterial or eukaryote origin of the DNA, it is imperative to further sequence the PCR fragments (Dierckens et al., 2009).

3.3.2 Hatchability

Disinfection of fish eggs may exert a negative impact on the hatchability, due to the death of the embryo or it being hindered to penetrate the egg shell (e.g. Arimoto et al., 1996; Mimura et al., 1998; Battaglene & Morehead, 2006). To assess the tolerance of fish eggs to specific disinfectants, the most important and easiest parameter to evaluate, is hatchability. However, not all studies mentioned the hatchability results in an unambiguous way (Shaw, 1957; Douillet & Holt, 1994; Cipriano et al., 2001), hereby missing the opportunity to indicate the specific tolerance of the tested fish species which would enable a comparison between teleosts.

The hatchability can be measured in three different ways. Firstly, all the remaining larvae can be counted and compared with the initial number of eggs that were disinfected, which engenders the hatching percentage (e.g. Verner-Jeffreys et al., 2007; Can et al., 2012; Wagner et al., 2012b; 2012c).

Although this technique offers the most precise results, it is very time consuming and virtually impossible in large scale experiments. Secondly, the hatchability can be determined by taking a water sample of the experimental tank, counting all dead and alive eggs or larvae in this sample and extrapolating this number per volume to the experimental tank (e.g. Rach et al., 1997; Stuart et al., 2010). This method is much quicker but caution is needed. To ensure a correct extrapolation, the eggs or larvae must be uniformly dispersed before sampling. Due to the phototactic reaction of larvae and the buoyancy characteristics of the eggs, this may be difficult to achieve. Thirdly, a small subsample of eggs can be held in a smaller tank or vial until hatching. Also here, the hatching percentage of this subsample is then extrapolated to the whole experimental unit (e.g. Salvesen et al., 1997). This technique requires an additional experimental set-up and the adoption of a smaller egg mass or water body may exert a non-negligible impact on hatchability.

3.3.3 Impact on post-hatching development

Besides envisaging hatchability as a parameter to assess the safety of a disinfectant, the effect on the hatching larvae themselves also needs to be determined. Indeed, in addition to inducing malformations of the fish larvae (Ben-Atia et al., 2007), the disinfection may also result in a reduced growth rate and diminished tolerance to stress, warranting the need to include these as safety criteria. Furthermore, to be able to fully characterize the safety profile of a disinfectant, it is imperative to evaluate long term effects by observing the larvae for a longer period. This gives an indication of possible differences in larval quality that are not expressed as mortality or growth retardation during early larval stages but may negatively impact juvenile and adult fish.

3.3.3.1 Malformations

Possibly elicited malformations may include an enlarged head, a curved spine, a different size/shape of the yolk sac or poor swim bladder inflation. These malformations may cause poor performance (Chatain, 1987) and when persisting in the adult stage, may lead to a reduced marketability (Bolker & Hill, 2000). Except for the studies of Ben-Atia et al. (2007) and Forneris et al. (2003), most experiments do not clearly state from what point onwards a certain aberration is defined as “malformation” (e.g. Douillet & Holt, 1994; Harboe et al., 1994; Hirazawa et al., 1999a; Grotmol et al., 2003; Ben-Atia et al., 2007; Wagner et al., 2012b). Depending on the study, larvae were monitored for a period between 2 (Stuart et al., 2010) and 100 days (Hirazawa et al., 1999a) post-hatching.

3.3.3.2 Growth rate

Douillet & Holt (1994) reported higher growth rates for larvae from disinfected fish eggs in addition to higher survival rates. Dabrowski et al. (2009) found significantly higher growth rates in 2 out of 6 replicates at 19 days after hatching (DAH) but lower growth rates were observed in 1 out of 6 replicates

after 27 DAH. In other studies where this criterion was investigated, no significant impact on the growth rate of the disinfected fish larvae was encountered (Hirazawa et al., 1999a; Stuart et al., 2010).

3.3.3.3 Stress tolerance

Salvesen and colleagues were the only researchers who tested stress tolerance of larvae hatched from fish eggs disinfected with glutaraldehyde. Both plaice (*Pleuronectes platessa*) (Salvesen et al., 1991) and turbot (*Scophthalmus maximus*) larvae (Salvesen et al., 1997) were exposed to seawater with very high salinity (50 vs 70 ppt). Turbot larvae were also evaluated for their tolerance to handling by placing the larvae in an Erlenmeyer and recording the mortality after 15 minutes of shaking (Salvesen et al., 1997).

3.4 DISINFECTANTS

In what follows, the most commonly used and upcoming disinfectants are discussed. Firstly, the mode of action is debated, followed by the range of concentrations and contact times tested, possible effects on bacterial/fungal load and potential impact on hatchability of disinfected eggs. Next to this, the effect of the developmental stage of the embryo during disinfection is listed. Finally, where available, results concerning long-term implications are mentioned.

As this article mainly focuses on disinfection of fish eggs, studies merely studying the tolerance of a species to the tested chemical, without providing data on fungal/bacterial load reduction, were only mentioned in the text and not included in the tables.

3.4.1 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is a widely used biocide that reacts as a strong oxidizing agent by producing hydroxyl free radicals (McDonnell & Russell, 1999). Oxidizing agents have the ability to remove electrons from a variety of molecules, including proteins, lipids and nucleic acids. The oxidation of these molecules causes major changes in morphological structure and function, triggering metabolic inhibition and cell death (Denyer & Stewart, 1998). Proteins, carbohydrates and lipids on the surface of microorganisms are easily accessible, and after disintegration of the outer structure, intracellular components including DNA and RNA can be oxidized.

H_2O_2 is considered environmentally friendly because it decomposes easily with the formation of oxygen gas and water (Kiemer & Black, 1997). It does not leave unwanted residues in tissues of living organisms or in the wastewater, making it a very suitable candidate for disinfection in a sustainable aquaculture setting. The activity of H_2O_2 is reduced in the presence of organic material or the enzyme catalase (Block et al., 2001). Organic matter interferes with the activity by providing a surface to adhere and therefore preventing contact of the disinfectant with the micro-organism one wants to incapacitate, by forming chemical bonds and hence inactivating the disinfectant or reacting chemically

with and neutralizing it. Another disadvantageous characteristic of H_2O_2 is that its addition to the water may induce a dramatic decrease of the pH, even more when the water hardness and concomitantly the buffering capacity of the water, is low. In contrast to fresh water, seawater has a high buffering capacity due to the large amount of minerals. Hence, especially in fresh water systems with soft water, it may be necessary to buffer the solution with $NaHCO_3$ to avoid a low pH (Heydarnejad, 2012; Wagner et al., 2010). Performing a water analysis therefore is crucial, with pH and water hardness as the most important parameters to monitor. This was done in most of the studies in which H_2O_2 was adopted as a disinfectant (Schreier et al., 1996; Rach et al., 1998; Peck et al., 2004; Verner-Jeffreys et al., 2007; Small, 2009; Wagner et al., 2010; Wagner et al., 2012b). When performing disinfection of fish eggs with H_2O_2, it is also necessary to take the water temperature into account. Indeed, an increased toxicity of H_2O_2 at higher temperatures has been suggested with the efficiency of H_2O_2 as a disinfectant possibly being negatively affected (Kiemer & Black 1997; Small, 2004).

In Table 3.2 a survey of the various studies using H_2O_2 is given, listing the fish species, adopted protocol, impact on hatchability and results in terms of bacterial/fungal load reduction. Studies were conducted regarding the effect of a single disinfection shortly after egg fertilization as well as disinfection on a regular basis. Single disinfections were performed with concentrations ranging from 0.005% (El-Dakour et al., 2015) to 3.5% (Verner-Jeffreys et al., 2007) and contact times between 1 (Wagner et al., 2010; 2012a) and 60 minutes (Kitancharoen et al., 1997a). A double disinfection with 1000 or 2000 mg/L for 2 minutes was tested by Wagner et al. (2012a). Disinfection on a regular basis was completed in flow-through systems, simulating the conditions in commercial hatcheries. Tested concentrations (Table 3.2) were much lower and ranged between 100 and 6000 µl/L, with a longer contact time of 15 (Waterstrat & Marking, 1995; Schreier et al., 1996; Barnes et al., 1998, 2003a, 2003b; Rach et al., 1998; Small & Chatakondi, 2006) to 60 minutes (Kitacharoen et al., 1997a). H_2O_2 was administered daily (Waterstrat & Marking, 1995; Barnes et al., 1998; Rach et al., 1998; Small & Chatakondi, 2006; Wagner et al., 2012b), every other day (Schreier et al., 1996) or twice a week (Kitancharoen et al., 1997a; Novakov et al., 2018) until hatching. Small & Wolters (2003) tested different H_2O_2 treatments (both single disinfections as well as daily flow-through treatments) but since all egg batches were treated with iodine in advance, comparison of the results with those of other studies is very difficult. No clear-cut conclusions regarding the optimal concentration and/or contact time are drawable, due to the large variety of applied concentrations and contact times in various fish species, and the adoption of various criteria to evaluate the safety and efficacy of the tested protocol.

Except for one tested protocol (15 g/L for 2 min) on rainbow trout (*Oncorhynchus mykiss*) eggs for which no significant impact was noted (Wagner et al., 2010), all tested procedures elicited at least a reduction in bacterial and fungal load. Douillet & Holt (1994) were the only ones succeeding in

obtaining red drum (*Sciaenops ocellatus*) eggs treated with 3% H_2O_2 for 5 min. An attempt by this research group to apply the protocol to other fish species failed in obtaining sterile and hatching eggs, indicating the large interspecies differences. The procurement of sterile fish eggs as a result of this protocol accentuates its value for developing axenic larval models in fundamental research. However, no data on the axenity of the post-hatching larvae were included. Further elaboration is warranted on the possible extrapolation of this modus operandi to other fish species, with the necessary adjustments in terms of concentration and contact time, to safeguard a consensus between sterility and hatchability. Situmorang et al. (2014) created a gnotobiotic model of Nile tilapia (*Oreochromis niloticus*) by combining 2% H_2O_2 for 10 minutes with 75 mg/L sodium chlorite for 2 minutes and an antibiotic mixture (Table 3.2). Axenity of the larvae was achieved in 1 out of 2 experiments with no negative impact on the hatching percentage. This disinfection protocol was also used in the follow-up study (Situmorang et al., 2016). Complete elimination of the fungi associated with the fish eggs was established in only two studies (Rach et al., 1998; Small, 2009).

For most of the tested treatments, hatching was similar to the eggs that remained untreated or even increased. A negative impact on hatching was only observed when the highest concentrations or contact times of a range of protocols were adopted (Kitancharoen et al., 1997a; Small & Wolters, 2003; Small & Chatakondi, 2006; Verner-Jeffreys et al., 2007; Can et al., 2010; El-Dakour et al. 2015). Also here, a large interspecies dissimilarity in tolerance was found (Table 3.2). It is also important to note that disinfection with H_2O_2 can cause changes in the egg morphology (Stephenson et al., 2005). These changes could interfere with hatching or normal embryonic development. Within one species, a high interbatch variation was noted with the disinfection of heavily colonized batches needing double concentrations of H_2O_2 compared to batches that were less colonized by bacteria (Verner-Jeffreys et al., 2007). Nevertheless, most studies did not test the protocols on different egg batches which may question their reproducibility in view of the above. No abnormalities in terms of both behavioral and morphological characteristics of newly hatched sea bream (*Sparus aurata*) and red porgy (*Pagrus pagrus*) larvae were encountered (Can et al., 2010).

Although most studies did mention the stage at which the eggs were disinfected, only a few investigated the effect of H_2O_2 on more than one developmental stage. Peck et al. (2004) found a significant interaction between treatment and developmental stage for cod, but not for haddock (*Melanogrammus aeglefinus*). Indeed, more developed cod eggs hatched better after disinfection with H_2O_2 while this was not the case for the early egg stages. For rainbow trout, the opposite was noted, whereby eyed embryos were more sensitive to disinfection with H_2O_2 than embryos closer to fertilization (Wagner et al., 2010). H_2O_2 disinfection prior to water hardening decreased survival in snapper (*Chrysophrys auratus*) eggs compared to disinfection afterwards (Partridge et al*.,* 2017). The

results of these three studies underscore the importance of including different developmental stages when conducting research on the effects of disinfectants and not extrapolating the results obtained in one fish species to another.

No increase in deformities in 8 or 10 days old larvae were noted following disinfection with H_2O_2, a finding which contributes to its potential as a good disinfectant (Douillet & Holt, 1994; Wagner et al., 2010). Normal or increased larval survival was encountered for 3 day old larvae (Verner-Jeffreys et al., 2007; El-Dakour et al., 2015). Also higher survival and growth rates were observed in 10 day old larvae after H_2O_2 disinfection (Douillet & Holt, 1994).

3.4.2 Glutaraldehyde

Glutaraldehyde is a commonly used dialdehyde, crosslinking proteins in the outer layers of the bacterial and fungal cells, which causes metabolic and replicative inhibition. When glutaraldehyde enters the environment, it is most likely to remain in the aquatic compartment but due to its high water solubility, the affinity to bioaccumulate is low. Glutaraldehyde is considered readily biodegradable in fresh water systems and potentially biodegradable in the marine environment based on the OECD criteria (Leung, 2001). In contrast to some other disinfectants, glutaraldehyde retains a high degree of activity in the presence of organic matter which is commonly present in batches of fish eggs (Fraise et al., 2004). The pH of the water impacts both the biocidal activity and the rate of reaction of glutaraldehyde, whereby an alkaline pH increases the biocidal activity drastically. Increasing the pH also leads to a more rapid bactericidal effect (Denyer & Stewart, 1998; Fraise et al., 2004; Rutala & Weber, 2008). The activity of aldehydes also increases largely with rising temperature (Gardner & Peel, 1986; Planas & Cunha, 1999) and this should be kept in mind when comparing disinfection protocols in between species requiring a different water temperature. Concerning human health, glutaraldehyde may be dangerous for the researchers and aquaculturists performing the disinfection as it may cause occupational asthma (Cullinan et al., 1992; Chan-Yeung et al., 1993; Gannon et al., 1995) or skin and eye irritation (reviewed in Takigawa & Endo, 2006) when control measures such as adequate ventilation are not or insufficiently implemented. The median lethal concentration (concentration that is lethal to 50% of the embryos, LC_{50}) of glutaraldehyde is similar for both warm water and cold water species and slightly higher for fresh water than to marine fish (Leung, 2001).

Glutaraldehyde is especially employed for its bactericidal and virucidal effect, although it also exhibits fungicidal properties. Table 3.3 lists the various studies that used glutaraldehyde, indicating, in addition to concentration and contact time, the effect on hatchability and bacterial/fungal load. The used glutaraldehyde concentrations range between 25 ppm (Cline & Post, 1972) and 3200 ppm (Morehead & Hart, 2003) for contact times of 2 (Escaffre et al., 2001; Can et al., 2010) to 60 minutes (Marking et al., 1994). Except for the studies of Forberg et al. (2011), Hansen & Falk-Petersen (2001), Pavlov &

Moksness (1993) and Schaeck et al. (2016a) all disinfections were performed with one single dose of glutaraldehyde (Table 3.3).

For most of the tested protocols, a reduction in the bacterial load was observed, which is sufficient in an aquaculture environment (Table 3.3). Regular disinfections with 600 mg/L glutaraldehyde could also protect the shell of common wolffish (*Anarhichas lupus)* eggs from bacterial destruction due to *Flexibacter* sp. and consequently dead or premature hatching could be avoided (Pavlov & Moksness, 1993). Sterility was achieved in part of the eggs in the studies of Can et al. (2010), Morehead & Hart (2003) and Salvesen et al. (1997), which is a necessary prerequisite in developing a gnotobiotic model. For the latter, different glutaraldehyde treatments were combined with the administration of 10 mg/L of both rifampicin and ampicillin to the hatching (Forberg et al., 2011; Schaeck et al*.,* 2016a) and rearing water (Dierckens et al., 2009). These latter two studies are part of the few that succeeded in producing bacteria-free eggs and larvae, however, both use antibiotics following disinfection. The resulting gnotobiotic models have been extensively used in larval research (Rekecki et al., 2009; 2012; 2013; Li et al*.,* 2014a; 2014b; Schaeck et al*.,* 2016b; 2017; Yaacob et al*.,* 2017; 2018; Aerts et al*.,* 2018; Nikolakakis et al*.,* 2018; Reyes-Lopez et al*.,* 2018). The fungicidal effect of glutaraldehyde on trout eggs was tested by Cline & Post (1972). Although a reduction in *Saprolegnia* infection could be observed, a negative impact on the hatchability was noted.

The resilience of the eggs to glutaraldehyde has been proven to be very species specific. In most species, high concentrations and contact times were used, without affecting the hatchability (Table 3.3). Concentrations without negative impact on hatching and larval survival range from 100 ppm for 5 minutes (red porgy and white sea bream; Katharios et al., 2007) to 800 ppm for 10 minutes (cod (Overton et al., 2010) and halibut (*Hippoglossus hippoglossus,* Salvesen et al., 1997). The difference in tolerance between various species may in addition be influenced by the water temperature. When comparing Atlantic halibut and turbot, the latter was found to be much more sensitive to glutaraldehyde (Salvesen et al., 1997). Also when spotted wolffish eggs (*Anarhichas minor*) were disinfected, survival depended strongly on the temperature at which glutaraldehyde was applied (Hansen & Falk-Petersen, 2001).

Escaffre et al. (2001) incorporated multiple developmental stages of Gilthead seabream hereby looking for differences in sensitivity to glutaraldehyde depending on the age of the embryo. Embryos displayed a very high mortality when disinfected younger than the blastopore closure stage. Once the embryos were older, hatching rates similar to those of the control group were obtained. This stresses the importance of incorporating different developmental stages when one wants to fully assess the impact of glutaraldehyde disinfection for a certain fish species.

For halibut eggs, none of the tested treatments (Table 3.3) showed a difference in survival or number of deformed larvae during the first stage (yolk-sac period). However, during first feeding the survival was found to be significantly higher for the eggs exposed to 400 ppm glutaraldehyde compared to 800 ppm and the untreated control group (Harboe et al., 1994). Striped trumpeter eggs (*Latris lineata*) displayed a normal hatching percentage when treated with 200 ppm and 400 ppm. Survival after 5 days was higher than the control group, but similar between both treatments. After 9 days, survival of the larvae treated with 400 ppm glutaraldehyde was significantly higher than that of the larvae disinfected with 200 ppm glutaraldehyde (Morehead & Hart, 2003).

Salvesen and colleagues were the only researchers to test stress tolerance of larvae hatched from fish eggs disinfected with glutaraldehyde. Both plaice (Salvesen et al., 1991) and turbot larvae (Salvesen et al., 1997) were exposed to seawater with very high salinity (50 and 70 ppt). For plaice, the time that the larvae survived the stress conditions was monitored. For turbot, the time until 50% of the larvae died was recorded. Plaice larvae that hatched from eggs disinfected with ≥ 800 ppm glutaraldehyde were significantly less tolerant to high salt concentrations. Turbot larvae from eggs treated with 400 or 800 ppm for the shortest contact time (2.5 minutes compared to 5 minutes) performed best. Turbot larvae were also evaluated for their tolerance to handling by placing the larvae in an Erlenmeyer and recording the mortality after 15 minutes of shaking. Also here the shortest contact time (2.5 minutes) resulted in the best performance (Salvesen et al., 1997).

3.4.3 Ozone

Ozone (O_3) is a highly reactive molecule causing the formation of a range of oxidants in the water. Ozone is known to be a fungicidal, bactericidal, virucidal and sporicidal agent by reacting with cell wall lipids and enzymes causing loss of enzymatic activity and leakage of cell components (Fraise et al., 2004). It is suggested to be a good egg disinfectant as only short exposure times starting from one minute are needed and it rapidly breaks down hereby forming oxygen. The presence of organic material in the water significantly shortens the half-life of ozone which is normally about 15 minutes (Mendicino, 2001). As for most of the disinfectants, redundant organic material should therefore be removed prior to disinfection. This can be done by rinsing the eggs with (sea)water for small-scale experiments, or making sure a high water flow is maintained in large-scale situations. When ozone disinfection is used in recirculation systems, it is important to take into account that ozone-produced oxidants are possible stressors for other lifestages of fish. To exemplify this, juvenile turbot displayed histological and physiological alterations when chronically exposed to ozone-produced oxidants (Reiser et al., 2010; 2011). Ozone is less affected by pH and temperature than most other disinfectants (Block, 2001). In high concentrations ozone may cause irritation of the mucous membranes of eye,

nose and throat of the users (Fraise et al., 2004). Another disadvantage is the need for a special device to produce ozonated (sea) water in a safe and controlled manner.

With regard to the efficiency of ozone disinfection, very promising results were obtained. Complete sterility, based on bacterial culture techniques could be achieved for gilthead seabream, sea bass *(Dicentrarchus labrax*), common dentex (*Dentex dentex*) and red porgy within the tolerance range of the species under study (Table 3.4). Some research groups were only interested in the sensitivity of the species to different ozone treatments, hence not including bactericidal and fungicidal effect as an evaluation criterion (Grotmol et al., 2003; Battaglene & Morehead, 2006). Other studies only focused on the elimination of one specific pathogen. Subsequent to ozone disinfection, the growth of *Saprolegnia* on brown trout eggs was delayed up to a week, although no real quantitative data were given in the article (Forneris et al., 2003). The authors concluded that ozone treatment is a good alternative to formaldehyde to combat saprolegniasis.

Overall, fish eggs only tolerate low ozone concentrations. The multiplication of concentration and contact time, denoted as "CT", is mainly used as a measure to compare different treatments. When overviewing the results of the various performed studies, the ideal exposure time and concentration are species dependent (Douillet & Holt, 1994; Grotmol & Totland, 2000; Can et al., 2012). The maximum values that were tolerated, range between CT 2.4 mg min/L (Ben-Atia et al., 2007) and CT 4 mg min/L (Can et al., 2012), with two large exceptions. Turbot (Grotmol et al., 2003) and haddock (Buchan et al., 2006) were found to be highly resilient to ozone, with maximum CTs of 13.8 mg min/L and 30 mg min/L, respectively. Although CT may be a simple way to analyze and liken various protocols, the individual values for contact time and concentrations in themselves may exert a large impact. As an example, for trumpeter eggs, ozone toxicity is more dependent on concentration than duration of exposure. Hatching was significantly delayed or reduced at CTs of more than 2.5 mg min/L, except for the larvae treated with 1 mg O_3/L for 5 minutes (CT=5) (Battaglene & Morehead, 2006). Varying only one of the two parameters in a study, being the concentration (Buchan et al., 2006; Ben-Atia et al., 2007; Can et al., 2012) or the contact time (Ballagh et al., 2011), may influence the conclusion regarding the ozone toxicity markedly and should therefore be avoided.

Little is known about temperature-dependent differences in tolerance, because most of the studies only disinfect at the temperature related to the species of interest. At lower temperatures, mulloway larvae (*Argyrosomus japonicas* Temminck & Schlegel) experienced increased toxic effects of ozone (Ballagh et al., 2011), but no such differences were observed for gilthead sea bream (Ben-Atia et al., 2007).

When a decrease in hatching was observed, it was noted that in some cases the embryo grew further in the chorion but failed to hatch (Arimoto et al., 1996; Mimura et al., 1998). It is suggested that this effect could be a consequence of either the inhibition of the hatching gland by ozone, causing a failure to secrete the hatching enzyme, or ozone modifying the characteristics of the egg shell, making it more resistant to the hatching enzyme (Grotmol et al., 2003).

Only Battaglene & Morehead (2006) included five stages of embryonic development of striped trumpeter embryos. Using low concentrations of 0.5 mg/L for 1 minute did not result in a decreased hatching for any of the stages. Higher concentrations (2 mg/L for 1 minute) were only safe for three days old eggs.

Larval deformities were not observed in any of the studies, except for gilthead seabream, where significantly more deformities, as well as higher incidence of swimbladder inflation, were noted for all tested ozone concentrations (Ben-Atia et al., 2007). Larval survival was monitored in two studies (Grotmol & Totland, 2000; Ben-Atia et al., 2007). Both studies observed significantly higher larval survival rates in some of the ozone treatments tested.

3.4.4 Iodophors

Iodophors are complexes of iodine and a solubilizing carrier, which acts as a reservoir of active 'free' iodine (Gottardi et al., 1985). When iodophors are diluted in water, iodine is slowly released. The antimicrobial action of iodine is rapid even at low concentrations but the exact mode of action is not well known. Iodine can penetrate the cell and react with different molecules (in particular the thiolgroup of enzymes) which eventually causes cell death. As is the case with many other disinfectants, the activity of iodine in low concentrations is largely reduced by organic matter. As opposed to other disinfectants, iodine reacts over a wide pH range and exhibits a limited dependence on temperature (Fraise et al., 2004). Iodine also has a good pathogen/host differential of toxicity (Amend, 1974).

Table 3.5 gives a resume of multiple studies using iodophors as a disinfectant and indicates the fish species used, the protocol, the results concerning reduction in bacterial/fungal load and hatchability of the eggs. Single dose disinfections were carried out with concentrations ranging from 5 mg/L (Chalupnicki et al., 2011) to 7500 mg/L (Chalupnicki et al., 2011) with contact times varying between 4 (El-Dakour et al*.,* 2015) and 180 minutes (Dabrowski et al., 2009. Cipriano et al. (2001) and Jodun & Millard (2001) tested double dose disinfection. Additionally, Khodabandeh & Abtahi (2006) evaluated different concentrations using twice a day flush treatments (Table 3.5). Some studies evaluated different iodine disinfection protocols in combination with regular treatments with formalin as an antifungal agent (Barnes et al., 2003b; Pravecek & Barnes, 2005; Chalupnicki et al., 2011). In other

trials, the iodine disinfection was preceded by treatment with 400 mg/L tannic acid for 4 minutes (Dabrowski et al., 2009).

A decrease in bacterial or fungal load could be observed in all studies. Complete sterility was obtained for red seabream (*Pagrus major)* and spotted halibut (*Verasper variegatus)* eggs (Hirazawa et al., 1999a) after disinfection with iodine (Table 3.5). Full sterility of the disinfected eggs was also achieved for California yellowtail (*Seriola lalandi*) and white seabass (*Atractoscion nobilis*), but hatchability was extremely low (0-2%) (Stuart et al., 2010). Various studies specifically focused on the elimination of a bacterial pathogen by means of iodine disinfection. Atlantic salmon (*Salmo salar*) eggs were disinfected with 50 mg/L active iodophor for 30 minutes (Cipriano et al., 2001). After 90 minutes, the eggs were disinfected a second time. No *Aeromonas salmonicida* could be detected after the first disinfection round. In contrast to these *in vivo* trials, *A. salmonicida* was still detectable after the first disinfection treatment in *in vitro* trials (without eggs) if concentrations were 1.10^7 Colony Forming Units (CFU)/mL or higher before disinfection. No viable bacterial cells of *A. salmonicida* were retrieved following the second disinfection. The hatching rate of the eggs or possible negative effects on the larvae were not examined. Trout eggs can tolerate concentrations up to 0.05% and as only 0.0056% iodine was needed to eliminate *Bacterium salmonicida* in less than 10 minutes, this treatment was reported as safe (Gee & Sarles, 1942). Although no quantitative data were given, prolonged hatching at this concentration was mentioned. *A. liquefaciens* could be fully removed from eyed rainbow trout eggs without negative effects on hatching after disinfection with iodine concentrations of 1% for 10 minutes (McFadden, 1969). Likewise, *A. liquefaciens* was eliminated from largemouth bass eggs with concentrations of 150-200 ppm for 15 minutes. However, at the highest concentrations, hatching rates decreased significantly (Wright & Snow, 1975). Iodine concentration of 500 mg/L for 15 minutes could completely eliminate *Renibacterium salmoninarum* from the surface of coho salmon eggs (Evelyn et al., 1984). Additionally, concentrations of 250 ppm (15 to 120 minutes post and during water hardening) could be used without negative effects on the hatchability (Evelyn et al., 1986a). However, *R. salmoninarum* could still be detected within the eggs (Evelyn et al., 1984; 1986a). In order to test if *Flavobacterium psychrophilum* can be prevented from being vertically transmitted, povidone-iodine disinfection (50 ppm for 15 minutes) was tested for five salmonid species. Newly unfertilized eggs were immersed in a *F. psychrophilum* suspension and consequently disinfected with povidone-iodine before fertilization and/or during water hardening and/or after water hardening. It was shown that only disinfection of the unfertilized eggs resulted in a total clearance of *F. psychrophilum* from the surface and within the eggs (Kumagai & Nawata, 2010). When disinfection was performed following fertilization, *F. psychrophilum* was still detected inside the eggs. The impact of the disinfection on the occurrence of other bacterial species was not assessed (Kumagai & Nawata, 2010; Loch & Faisal, 2016; 2018).

Contrary to these results, Brown et al. (1997) showed *in vitro* that a minority of *F. psychrophilum* cells could survive 100 ppm iodine for 30 minutes. Also *Cytophaga psychrophila* could still be detected after iodine disinfection with concentrations ranging between 50 and 1000 ppm for 15 minutes (Kumagai et al., 1996). No possible impact on survival was mentioned.

The effect of iodine disinfection on hatching percentages varied greatly depending on the study. The large variety in species, tested concentrations and contact times make it virtually impossible to draw well-founded conclusions. Next to the studies dealing with the effect of iodine on the microbial load (Table 3.5), other studies focused only on the tolerance of the eggs towards this disinfecting agent. The latter was done for tilapia (*Oreochromis mossambicus*; Subasinghe & Sommerville, 1985), black sea turbot (*Psetta maxima*; Aydin et al., 2011), westlope cutthroat trout (*Oncorhynchus clarkii lewisi,* Pravecek & Barnes, 2003), Atlantic salmon (Jodun & Millard, 2001), yellow perch (*Perca Flavescens*, El-Gawad et al*.,* 2016), brown trout (*Salmo trutto*, Lahnsteiner & Kletzl, 2015), burbot (*Lota lota*, Lahnsteiner & Kletzl, 2015), European Grayling (*Thymallus thymallus*, Lahnsteiner & Kletzl, 2015) and 3 ornamental fish (Chambel et al*.,* 2014).

Also for iodophors, the developmental stage of the embryos at the moment of disinfection may have a large impact on the hatchability. Katharios et al. (2007) disinfected both 0- and 1-day old eggs of red porgy and white sea bream (*Diplodus sargus sargus*). In contrast to 1-day old eggs, 0-day old eggs showed a very high reduction in hatchability following iodine disinfection, but once hatched, no influence on larval survival was observed. It appears that eyed salmonid and burbot eggs have a high tolerance to iodophor disinfection compared to earlier stages and disinfection during water hardening should hence be avoided (Wagner et al., 2010; Lahnsteiner & Kletzl, 2015). This was not the case for Atlantic salmon (Chalupnicki et al., 2011) and brown trout (Strauss et al., 2016) egg, since none of the tested disinfection treatments during water hardening caused an impact on the hatchability. Spotted halibut eggs were best disinfected with iodine one day before hatching, upon initiation of the heartbeat. In red sea bream, the morula stage was the most suitable stage for disinfection (Hirazawa et al., 1999a). Although the hatchability of black sea turbot eggs was similar following disinfection at 0-day, 1-day and 2-days of age, when disinfection was performed at the latter two ages, a significantly higher number of abnormalities was noted (Aydin et al., 2011). Also grouper (*Epinephelus coioides*) eggs were more resilient to stress and hence disinfection during the eyed stage (Tendencia, 2001).

In addition to the developmental stage of the eggs, the physiological state of the broodstock fish may also have a marked effect on egg hatchability following iodine disinfection. A difference in iodine sensitivity between newly fertilized eggs stripped from different females of rainbow trout was observed by Alderman (1984). No clear explanation for these phenomena was given, but a difference

in the age of the eggs in the females before stripping or inequalities in egg membrane or amount of ovarian fluid may be put forward as potential explanatory hypotheses. Also Pravecek & Barnes (2003) observed significant differences in survival between replicates of eggs originating from different females. Hirazawa et al. (1999b) observed that survival was reduced in eggs released early during the spawning period. Eggs collected at the beginning of the spawning season were inferior in quality and therefore more sensitive to iodine disinfection.

To evaluate the long term impact of iodine disinfection, various studies monitored larval survival (Hirazawa et al., 1999a; Katharios et al., 2007; Dabrowski et al., 2009; Overton et al., 2010; Stuart et al., 2010; Wagner et al., 2010, Chalupnicki et al., 2011; El-Dakour et al., 2015; El-Gawad et al., 2016). The length of the period of monitoring ranges between two (Stuart et al., 2010) and 100 days (Hirazawa et al., 1999a). In none of the studies, long term effects were noticed. In addition to larval survival, the occurrence of deformities was checked in some studies (Hirazawa et al., 1999a; Overton et al., 2010; Wagner et al., 2010; 2012b; 2012c; Aydin et al., 2011; Lahnsteiner & Kletzl, 2015), as was the total length of the larvae at hatching (Stuart et al., 2010), at 19 and 27 days (Dabrowski et al., 2009) or at 80-100 days post-hatching (Hirazawa et al., 1999a). Also here, no significant impact was observed, except for black sea turbot eggs where concentrations of 1500 and 3000 ppm for 10 minutes caused significantly more abnormalities. Swimbladder inflation rates and larval weight were followed in Walleye larvae (*Sander vitreus*). No negative effects on swim bladder inflation could be noticed but in 2 out of 6 replicates, iodine treatment caused significant changes in larval weight (Dabrowski et al., 2009).

3.4.5 Formaldehyde

Formaldehyde is an extremely reactive chemical that interacts with proteins, DNA and RNA (Fraenkel-Conrat et al., 1945). User safety (IHCP, 2006) and the effect of effluents on the environment, especially in lacustrine ecosystems (Stuart, 1983), are concerns related to formaldehyde treatments. It is the most simple organic compound of the aldehydes, which are known to have an increased activity with raising pH and temperature (Schliesser & Wiest, 1979; Planas & Cunha, 1999). As for many other products, formaldehyde is less effective in the presence of protein organic matter (Fraise et al., 2004). Most of the time it occurs as solution containing 34-38% formaldehyde, called formalin. Formalin is reported to be effective against bacterial, fungal, parasitical and viral infections and is widely used for disinfection of fish eggs, as it was for a long period (1991-2007) the only fungicide approved by the United States Food and Drug Administration (FDA) (www.fda.gov; accessed 28th July 2014).

Table 3.6 gives an overview of the various protocols using formalin as a disinfectant of fish eggs, showing the fish species used, tested protocols, hatchability and noted fungal/bacterial load reduction. Both single dose and repeated disinfections were performed. For single dose disinfection,

concentrations ranging from 0.005% (El-Dakour et al., 2015) to 1% (Stuart et al., 2010) were used with contact times between 4 (El-Dakour et al., 2015) and 60 minutes (Stuart et al., 2010) (Table 3.6). Repeated disinfection protocols were tested in flow-through systems, simulating aquacultural conditions. For this, concentrations were much lower, and ranged between 500 (Barnes *et a*l., 2005; Schreier et al., 1996) and 7500 µl/L (Rach et al., 1997). These repeated disinfections were performed daily (Barnes et al., 2000; 2001; 2003a; 2003b; 2005; Small & Chatakondi, 2006; El-Gawad et al., 2016), twice a day (Barnes et al., 2003b; Khodabandeh & Abtahi, 2006), 3 or 4 times a day (Small & Chatakondi, 2006), every other day (Bailey & Jeffreys, 1989; Rach et al., 1997) or once a week (Wanatabe, 1940) depending on the study.

Except for Cline & Post (1972), all used concentrations caused a reduction or elimination of fungal and bacterial load (Table 3.6). Bacterial sterility was only achieved in one study, where 8 out of 25 trials for *Tilapia macrocephala* (Shaw, 1957) resulted in sterile eggs but only little information on hatchability and the long term effect of these treatments was given. Furthermore, complete fungal elimination was achieved in 2 studies (Cline & Post, 1972; Rach et al., 1997)

As with iodine, the results on hatchability were very variable, and no solid conclusion could be made. Hatchability decreased, increased or stayed normal after disinfection depending on the study. Also here, some studies only focused on the tolerance of eggs to a disinfectant rather than also looking at the effect on the fungal or bacterial load (Table 3.6). Tolerance studies were performed on landlocked Fall Chinook salmon (*Oncorhynchus tshawytscha*; Barnes et al., 2000; 2001; 2003a; 2003b; 2005), hybrid catfish (*Ictalurus punctatus* x *I. furcatus*; Small & Chatakondi, 2006), tilapia (Kitancharoen & Sommerville, 1985) and yellow perch (El-Gawad et al*.,* 2016).

Although Stuart et al. (2010) indicated the developmental stage at which disinfection was performed, as far as we know no study has been executed to investigate the difference in tolerance in between different developmental stages. Except for El-Dakour et al. (2015), Shaw (1957) and Stuart et al. (2010), all studies performed regular disinfection treatments (twice a day or every other day).

Following formalin disinfection, an increased larval survival at three days post hatching was observed in bluefin seabream (*Sparidentex hasta*, El-Dakour et al., 2015) and yellow perch (El-Gawad et al., 2016). At the first feeding stage, no lower long term survival rate nor smaller larval size were observed in California yellowtail, white seabass and California halibut (*Paralichthys californicus*) (Stuart et al., 2010). Also Forneris et al. (2003) did not note larval deformities associated with the disinfection protocol. These were the only studies giving any indication of deformities or possible long term effects.

3.4.6 Peracetic acid

Peracetic acid (PAA) is a peroxide of acetic acid and is available in an equilibrium mixture with acetic acid, hydrogen peroxide and water, whereby the combination of PAA and hydrogen peroxide works synergistically. Oxidising agents like H_2O_2 and PAA react strongly with thiol groups in enzymes, proteins and the cell membrane (Denyer & Stewart, 1998), increasing cell wall permeability (Kitis, 2004). PAA is known to be bactericidal, virucidal and fungicidal. Moreover, it decomposes to harmless compounds and forms almost no toxic byproducts (Kitis, 2004). PAA demonstrates antimicrobial activity in a wide temperature range (Schliesser & Wiest, 1979; Stampi et al., 2001), but it is affected by pH with decreasing activity in alkaline conditions (Sanchez-Ruiz et al., 1995). It remains effective in the presence of organic material. *In vitro* studies indicated that the use of peracetic acid (especially products with a low PAA: H_2O_2 ratio) can reduce growth of *F. columnare* and *Saprolegnia parasitica*, two major fish pathogens (Marchand et al., 2012), making it an interesting product to test *in vivo*.

As PAA has only recently been included in disinfection experiments, few studies have been performed (Table 3.6). Straus et al. (2012a) tested the fungicidal effect of PAA on channel catfish (*Ictalurus punctatus*) eggs with concentrations ranging from 2.5 to 20 mg/L, administrated twice a day in a flow-through system. A strong reduction in fungal growth was observed. The bactericidal effect of PAA was tested on cod eggs with two commercially available products (3.5 ml/L for 1 min (Brown et al., 2005); 1% for 60s (Treasurer et al., 2005)). For both products, sterile cod eggs were obtained following disinfection. Also for haddock, disinfection with the latter resulted in a major reduction in bacterial load, but no sterility was obtained.

Overall, good hatchability was observed for channel catfish and cod eggs after disinfection. For haddock, hatching rates were dramatically low (Table 3.6).

None of the studies mentioned different developmental stages nor the possible occurrence of deformities or long term effects on survival or development of the post hatching larvae.

3.4.7 Tannic acid

Tannic acid is a specific commercial form of tannin, a type of polyphenol that is extracted from plants. These natural substances are widespread and readily available in the environment (White, 1957) and commonly used as food additives. They are categorized as "generally recognized as safe" (GRAS) according to the Code of Federal Regulations (21CFR184.1097). Tannic acid has been used in aquaculture to eliminate adhesiveness of fish eggs, thereby facilitating the handling and chemical treatment (Rottmann et al., 1988; Bouchard & Aloisi, 2002; Walker et al., 2010). The presence of organic material is therefore not destructive for the activity of tannic acid. No clear data on the effect of the temperature and pH could be found. Although different *in vitro* studies have shown that tannic

acid can inhibit growth of bacterial species including *F. columnare* and *Edwardsiella ictaluri* (Chung et al., 1995; Zhao et al., 1997; Schrader, 2008), only one study has performed *in vivo* disinfection studies of fish eggs adopting tannic acid (Table 3.6).

In this study, eggs of rainbow trout were disinfected with tannic acid concentrations of 0.0002%, 0.002% and 0.02% for contact times of 5 or 15 minutes. A reduction in bacterial load was observed, but tannic acid was less effective than iodine in reducing bacterial growth (Wagner et al., 2012c). Because none of the treatments caused significant differences in hatching success or the occurrence of deformations or dead larvae (2 days post hatching), there might be room for improvement by increasing the concentration and/or contact time. Cornwell et al. (2011) observed that when tannic acid was combined with iodine, tannic acid destroyed the effectiveness of both products. Since the eggs in the study of Wagner et al. (2012c) were treated with iodine in the hatchery before disinfection, residues might have been present in the environment, which could have influenced the bactericidal effect of the tannic acid.

3.4.8 Bronopol

Bronopol is a broad spectrum bactericide, which is frequently used in food production and water disinfection. The product is believed to be toxic to bacteria due to its catalytic oxidation of accessible thiol groups, especially in the cytoplasmic and membrane-bound enzymes, causing metabolic inhibition (Shepherd et al., 1988; Denyer & Stewart, 1998). Bronopol is considered environmentally safe as it has a short lived environmental persistence and is very unlikely to bioaccumulate. However, it is toxic to marine and fresh water fish, therefore industrial effluent discharges needs to be highly controlled (US EPA, 1995). The activity of bronopol increases with increasing pH and temperature (Shepherd et al., 1988). No information concerning the effect of organic matter on the efficiency of bronopol was found.

Birkbeck et al. (2006) tested the bactericidal effect of bronopol *in vitro* for 13 bacterial strains of which 10 were found on eggs of Atlantic cod and halibut. Minimal inhibitory concentrations of 64 µg/ml inhibited growth of all species, but for killing the bacterial strains higher concentrations were needed. A low bactericidal effect with bronopol concentrations ranging from 50 to 450 ppm was also observed by El-Dakour et al. (2015). Apart from these *in vitro* tests, Treasurer et al. (2005) also investigated the *in vivo* effect of bronopol on eggs of both cod and haddock. First, single dose disinfection was tested. For cod, tested concentrations varied between 0.05% and 1% bronopol for 45 seconds, whereas for haddock, only 0.05% and 0.5% for 60 seconds were tested. In addition to single dose disinfection, also different daily drip regimes for haddock were investigated. The possible fungicidal effect of bronopol was tested on brown trout eggs. Daily administration of 50 mg/L entrained an almost complete

elimination of *Saprolegnia* and increased hatching. However, no long term effects were investigated (Aller-Gancedo & Fregeneda-Grandes, 2007).

For single dose disinfection, all treatments caused a reduction in bacterial load but sterile eggs could only be obtained for 2 treatments of cod eggs (0.5% and 1%). After disinfection, cod eggs showed significantly higher survival to hatch for the protocols adopting 0.05% and 0.5% bronopol. For haddock eggs, no such difference was observed. Normal hatching and larval survival rates were observed for bleufin seabream eggs following bronopol disinfection (El-Dakour et al., 2015).

For daily drip disinfection, significantly higher hatching rates could be observed when eggs were daily exposed for 30 minutes with 0.05% bronopol. On incubation day 9, no significant differences in larval appearance after egg disinfection were noted (Treasurer et al., 2005).

3.4.9 Sodium chloride

Sodium chloride has been used to control or prevent fungal contamination for several decades. The product achieves its antifungal properties by increasing the osmotic pressure and changing the water uptake of the microorganisms (Anbalagan et al., 2013). No information on the effect of organic load, changing pH or water hardness was found. As it is harmless to the environment and human health, sodium chloride has been granted low regulatory priority drug status by the United States Food and Drug Administration (FDA)(Schreier et al., 1996).

Table 3.6 lists the studies using sodium chloride for its fungicidal effect. Next to concentration and contact time, also the effect on hatchability and fungal load were mentioned. For single disinfection treatments, tested concentrations range between 25 ppm (Rasowo et al., 2007) and 50 ppt (Kitancharoen et al., 1997b) for contact times between 15 (Marking et al., 1994; Waterstrat & Marking, 1995; Schreier et al., 1996) and 60 minutes (Marking et al., 1994; Froelich & Engelhardt, 1996; Kitancharoen et al., 1997b).

Frequency of treatment varied greatly. Sodium chloride was administered once (Marking et al., 1994; Kitancharoen et al., 1997b; Rasowo et al., 2007), daily (Waterstrat & Marking, 1995; El-Gawad et al., 2016), twice a day (Khodabandeh & Abtachi, 2006), every other day (Schreier et al., 1996), twice a week (Kitancharoen et al., 1997b; Novakov et al., 2018) or continuously (Kitancharoen et al., 1997b).

For most of the tested protocols, a reduction in fungal infection was noticed. Infection of *Saprolegnia parasitica* could be completely avoided for common carp (Khodabandeh & Abtachi, 2006) and rainbow trout eggs (Kitancharoen et al., 1997b) after treatment. Although a fungicidal effect was observed, all concerned studies, achieved better results with other disinfectants. However, its low cost price and user safety speaks in favor of this product (Marking et al., 1995; Waterstrat & Marking, 1995; Schreier

et al., 1996; Khodabandeh & Abtachi, 2006; Rasowo et al., 2007). Salt treatment was also preferred over malachite green to control fungal infestation as salmon larvae originating from salt-treated eggs showed less malformations (Edgell et al., 1993).

Hatchability was affected in different ways depending on the concentrations used. For common carp, hatchability was significantly higher for all tested concentrations (Table 3.6) except the highest (35,000 mg/l sodium chloride added for 15 minutes twice a day; Khodabandeh & Abtachi, 2006). In contrast, longer durations (a single disinfection of 60 minutes) were toxic to common carp eggs at, concentrations of only 5,000 mg/l (Froelich & Engelhardt, 1996). Other species were much more sensitive. African catfish eggs (*Clarias gariepinus*) hatched significantly more when treated with concentrations between 100 and 1000 ppm but when concentrations increased to 10,000 ppm, hatchability was severely compromised (Rasowo et al., 2007). No significant difference in egg mortality could be observed for Fall Chinook Salmon after daily disinfection with 30,000 ppm sodium chloride for 15 minutes (Waterstrat & Marking, 1995). For rainbow trout eggs, disinfection with concentrations ranging from 15 to 30 ppt for 1 hour could be performed without significant reduction of the survival. Higher concentrations increased embryonic mortality (Kitancharoen et al., 1997b). Also repeated disinfection with 500 or 1000 mg/L daily (El-Gawad et al., 2016), 30 ppt for 15 minutes every other day (Schreier et al., 1996), twice a week with concentrations of 15 to 25 ppt (Kitancharoen et al., 1997b) or 1 to 2.5% (Novakov et al., 2018) and continuous treatment with concentrations between 3 and 7 ppt resulted in lower fungal infection rates without negative effects on the hatching results (Kitancharoen et al., 1997b).

As mentioned before, only four studies performed one single disinfection. Except for Kitancharoen et al. (1997b), none of the researchers mention the exact developmental stage at which disinfection took place nor investigated a possible difference in tolerance between different developmental stages. When regular disinfections were performed, some studies do mention the developmental stage at which the disinfection protocol was stopped. This could be the onset of the eyed stage (Khodabandeh & Abtachi, 2006) or the start of hatching (Schreier et al., 1996; Kitancharoen et al., 1997b). No monitoring of long term effects was indicated.

3.4.10 Copper sulphate

Copper sulphate is mainly used as algaecide and to treat parasites in aquaculture (Wagner & Oplinger, 2013). Although different *in vitro* studies have been performed (Straus et al., 2009a; Wagner & Oplinger, 2013; Sun et al., 2014), only few studies evaluated copper sulphate as an egg disinfectant. Copper has an oligodynamic effect towards bacteria. Although the mechanism is still not completely understood, it is suggested that copper ions distort the cell wall by binding to negatively charged parts of the membrane (Shrestha et al., 2009). Copper also inhibits certain enzymes and reduces defense

mechanisms against free oxygen radicals (Stauber & Florence, 1987). It leads to a reduced uptake of Ca^{2+} and higher loss of Na^{+} in zebrafish (*Danio rerio* Hamilton) (Alsop & Wood, 2011). The level of toxicity of copper depends on the water chemistry. Trivalent metal ions (such as Fe and Mn) result in a lower effectiveness (Stauber & Florence, 1987). The total alkalinity, the total hardness (measured as $CaCO_3$), salinity and pH of the water have an influence (Wagner & Oplinger, 2013). Soft acid water renders copper much more toxic compared to hard alkaline water (Howarth & Sprague, 1978). However, alkalinity and hardness play a minor role when treating eggs as the contact time is long (Straus et al., 2009b). As copper ions bind to all negatively charged particles, water with a high organic load leads to less ions available to bind to fungi or bacteria (Straus et al., 2009b, Wagner & Oplinger, 2013).

Table 3.6 lists the different studies that used copper sulphate, marking in addition to the concentration and contact time, the effect on hatchability and possible reduction in bacterial or fungal load. Across all studies, used concentrations range between 2.5 mg/L (Small & Chatakondi, 2006; Straus et al., 2009b) and 700 mg/L (Wagner & Oplinger, 2013). Single disinfections were performed with contact times of 2 (Wagner et al., 2012a) or 15 minutes (Wagner & Oplinger, 2013). In all other studies chemicals were administrated in a flow-through system and disinfection was done twice (24 and 48 hours after harvest, Wagner et al., 2012a), three times (Strauss et al., 2016), on a daily basis (Small & Chatakondi, 2006; Straus et al., 2009b; 2009c; 2012b; Mitchell et al., 2010) or every other day (Wagner et al., 2012a).

For all tested protocols a significant reduction in fungal load was observed (Mitchell et al., 2010; Straus et al., 2009b; 2009c; 2012b; Wagner et al., 2012a). Also a reduction in bacterial load (*F. psychrophilum*) could be achieved but effective concentrations exceeded tolerance levels for rainbow trout eggs (Wagner & Oplinger, 2013).

The limited number of studies evaluating copper sulphate as an egg disinfectant makes it difficult to draw species specific conclusions (Table 3.6). For channel catfish, maximum tested concentrations did not exceed 100 mg/L, without affecting hatching (Straus et al., 2012b). For hybrid catfish, concentrations up to 10 mg/L were tested, without negative effects on hatchability (Small & Chatakondi, 2006) but for rainbow trout, decreased hatching was observed for concentrations of 500 mg/L or more (Wagner & Oplinger, 2013). A significant increase in hatching was only observed in leatherside chub eggs (*Lepidomeda copei*) after disinfection with 40 or 60 mg/L for 2 minutes (Wagner et al., 2012a) and in sunshine bass eggs (*Morone chrysops* x *M. saxatilis*) following disinfection with 20 or 40 mg/L copper sulphate (Strauss et al., 2016)

All studies stopped disinfection protocols at the onset of the eyed stage of the embryo because the copper sulphate may be toxic to the newly hatched larvae (Straus, 2008). For sunshine larvae, the LC_{50} (lethal concentration resulting in 50% mortality) for 24h (5,4 mg/L copper sulphate) and for 48h (3.9 mg/L copper sulphate) was determined (Strauss et al., 2016). None of the studies compared the effect of copper sulphate on different developmental stages. Premature hatching was evaluated by Straus et al. (2009b) but no significant dose effect could be observed. Following disinfection, copper sulphate has been taken up into the egg chorion, which might extend the fungicidal effect and possibly its toxicity. However, egg disinfection with 20 mg/L did not result in an increased amount of larval deformities (Strauss et al., 2016). No additional studies included long term effects.

3.4.11 Antimicrobial agents

To enable understanding the host-microbe interactions in fish larvae without interference of unknown microbiota, a gnotobiotic larvae model is warranted. Developing such a model requires fully sterile fish eggs as a starting point. Because of the difficult and delicate balance between sterility and hatchability, only a handful of gnotobiotic models were developed with all but one (zebrafish; Rawls et al., 2004) adopting antimicrobial agents in the hatching and sometimes rearing water (Dierckens et al., 2009; Forberg et al., 2011; Situmorang et al., 2014). Although antimicrobial agents did not seem to hamper the hatchability of the eggs (Munro et al., 1995; Dierckens et al., 2009; Forberg et al., 2011; Situmorang et al., 2014), caution is needed. Penetration of the antibiotics through the egg wall has been recorded. However, no antibiotics could be detected after 1 to 7 days after administration (Evelyn, 1986b). Researchers should strive to completely remove the antimicrobial agents before the start of an experiment to avoid unwanted and unknown interactions with the target organism (Marques et al., 2006). Furthermore, if the antimicrobial agents are not removed, the envisaged microorganisms need to be made resistant to the used antimicrobial agent by multiple *in vitro* passages, which may have an impact on certain traits of the micro-organism under study (Fux et al., 2005).

Four different antibiotic mixtures were used to disinfect fish eggs in order to obtain bacteria-free larvae. Turbot eggs were incubated in an antibiotic solution containing 10 µg/ml oxolinic acid, 10 µg/ml kanamycin, 10 µg/ml erythromycin, 150 µg/ml penicillin G and 75 µg/ml streptomycin for 24h. After incubation, the eggs were rinsed with sterile seawater. With this protocol, different bacteria-free flasks (each containing > 30 eggs) could be accomplished (Munro et al., 1995). No data on hatchability were given, but survival after hatching seemed to be normal. Verner-Jeffreys et al. (2003) likewise used this antibiotic solution for incubation of halibut eggs, additionally including glutaraldehyde (prior to the antibiotic solution) and PAA (following the antibiotic solution) to disinfect. Also here, the eggs were rinsed before hatching and larvae were kept in sterile water without additives. However, only 1 bottle was found to be sterile before bacterial inoculation. A second antimicrobial mixture containing 100

µg/ml ampicillin, 5µg/ml kanamycin and 250 ng/ml amphotericin B was used by Rawls et al. (2004) on zebrafish eggs. Also here a limited incubation period was adopted. After 6h, the eggs were rinsed and left to hatch in sterile water. Complete sterility was obtained. The resulting protocol was further adopted by Pham et al. (2008), Caruffo et al. (2016), Sheng et al. (2018) and slightly modified by Oyarbide et al. (2015) and Pérez-Ramos et al. (2018) to generate gnotobiotic zebrafish. A third antimicrobial mixture containing 10 mg/L rifampicin and 10 mg/L ampicillin was used by Dierckens et al. (2009) and Forberg et al. (2011) to obtain bacteria free sea bass and cod larvae, respectively. Whereas Dierckens et al. (2009) permanently housed the sea bass larvae in this antimicrobial solution, Forberg et al. (2011) transferred the cod larvae to sterile seawater without additives at the moment 50% of the larvae were hatched. Hatchability was not reduced. The fourth antimicrobial mixture consisted of 10 mg/L ampicillin, 10 mg/L rifampicin, 10 mg/l trimethoprim, 10 mg/L gentamycin and was combined with the antifungal products Amphotericin-B (0.5 mg/L) and Fluorescent Brightener (25 mg/L). These products were added to the water used during the 24 h between two disinfections (2% H_2O_2 for 10 minutes and 75 mg/L sodium hypochlorite for 2 minutes) and after disinfection during the rest of the experiment. In one out of two experiments, bacterial contamination was observed. No negative impact of the disinfection procedure on the hatching percentage was noted. Only one developmental stage was included and long term effects were not examined (Situmorang et al., 2014).

In addition to facilitating the development of a gnotobiotic model, antimicrobial agents were also tested as disinfectants against specific pathogens. Erythromycin was evaluated in different concentrations before and during water hardening to eliminate *R. salmoninarum* on the surface and within salmonid eggs without good results (Bruno & Munro, 1986; Evelyn et al., 1986c). Also penicillin G and proflavin did not eliminate *R. salmoninarum* on salmonid eggs (Bruno & Munro, 1986). None of the tested treatments caused a reduction in hatching rates. Treating rainbow trout eggs with streptomycin or tetracycline significantly reduced the amount of *F. columnare* with increased hatching rates as a result. However, the used concentrations were not mentioned (Barnes et al., 2009). Furthermore, penicillin and streptomycin treatment reduced *F. psychrophilum* in rainbow trout eggs without negative effects on hatching and larval deformity rates (Oplinger et al., 2015). Penicillin-G (10000 IU/ml) resulted in no significant effect on hatching of rainbow trout eggs nor amount of larval deformities (Wagner et al., 2012d).

3.5 CONCLUSION

Various methods are used to verify the efficiency of disinfectants. Even within the culture based techniques, different culture media, incubation temperatures and times are employed in addition to the diversity in sampling protocols. Some research groups fortified their claim of bacterial and/or

fungal load reduction by adopting non-culture based techniques to evaluate the presence of non-culturable bacteria. Also for the latter, a difference in approach is noted. This myriad of techniques and protocols makes it very difficult to make a comparison regarding efficiency in between disinfectants. Hence, there is a serious need for a standardized protocol to verify the disinfection and/or sterilization potential of disinfectants listing the criteria that need to be met before claims can be made in this domain. Researchers should therefore strive to combine culture dependent and culture independent techniques. In any case, this will result in the obtainment of the most reliable outcomes when striving for fully sterile eggs or an accurate determination of decrease in microbial load.

Next to the disinfection efficiency, also the impact of the disinfectant on the fish egg, hatching and developing larva and older life stages needs to be evaluated when one aims for a full safety profile. Also here, no uniform protocol has been used. Determining the effect on hatchability is a minimal requirement for tolerance studies. Using a uniform way of measuring this parameter would enhance comparison between studies. Additionally, some studies use categorical values to describe the hatching results, rendering it difficult to compare results. Next to hatchability, other criteria warranting examination include malformations, growth, short- and long-term survival and stress tolerance. Indeed, various studies have demonstrated a potential impact of disinfectants on these parameters. Again, including the possible differences in tolerance of different developmental stages as well as taking into account the impact on post-hatching development, both in a standardized way, would provide a marked added value. As already mentioned, water parameters (amongst others temperature, pH, water hardness) should at least be indicated to be able to seize their potential impact on the efficacy and/or safety. Taking this further, testing the impact of changes in these parameters on the efficiency of and the tolerance of the species towards the disinfectant, would greatly enhance our knowledge on the disinfectant in question and equally important, enable their mutual comparison for the fish species of interest.

Although these were only briefly touched upon in this review, besides efficacy and safety for the teleost egg, user safety and environmental impact naturally are as equally important parameters and need to be included when generating a full data-set linked to a specific disinfectant. As reviewed for salmon aquaculture, there is little transparency about the use and dosage of the different disinfectants and insufficient research concerning the environmental impact of adopted chemicals has been done so far (Burridge et al., 2010).

In view of the above, this review is to be regarded as a plea for developing and/or pinpointing standardized yet feasible protocols to evaluate both the efficacy and safety of disinfectants and

endorsing these in further research into the use of egg disinfectants in sustainable aquaculture as well as research settings.

3.6 ACKNOWLEDGEMENTS

Prof. dr. Paul Simoens is gratefully acknowledged for critically reviewing the manuscript. Financial support by the Special Research Grant (Bijzonder Onderzoeksfonds, BOF12/GOA/022) of Ghent University, Belgium is gratefully acknowledged.

Table 3.1 - Overview of studies in various fish species using culture dependent and/or culture independent techniques to evaluate the effect of a specific disinfectant on the bacterial load. For the culture dependent techniques, the incubation medium, incubation time and temperature are listed.

References	Culture dependent			Culture independent	Fish species
	Medium	**Temperature**	**Incubation time**		
Barnes et al. 2005	Tryptic glucose extract agar (TGE)	20°C	3 days	SEM quantification of surface coverage	*Oncorhyncus tshawytscha*
Ben-Atia et al. 2007	Tryptic Soy Agar (TSA) with salt (final concentration 15g/l)	25°C	6 days		*Sparus aurata*
Brown et al. 1997	Tryptone Yeast Extract (TYE) broth	17°C	72 hours		*Oncorhynchus mykiss*
	followed by TYE plates (*F. psychrophilum*)	17°C	72 hours		
Can et al. 2010	TSA, Thiosulfate-citrate-bile salts-sucrose agar (TCBS)	20°C	3 days		*Dentex dentex, Pagrus pagrus, Sparus aurata*
Can et al. 2012	TSA, TCBS both supplemented with 1.5% NaCl	20°C	3 days		*Dentex dentex, Dicentrarchus labrax, Pagrus pagrus, Sparus aurata*
Chalupnicki et al. 2011	0.1% peptone:0.05% yeast extract dilution followed by Reasoner´s 2A agar plates	20°C	48-72 hours		*Salmo salar*
Cipriano et al. 2001	Tryptic soy broth (TSB)	18-20°C	18 hours		*Salmo salar*
	=> dilution plating on CBB (TSA+ 0.1% Coomassie Briljant Blue) for *A. salmonicida*	18-20°C	72 hours		
Dierckens et al. 2009	10% Marine Broth (MB)+ 15% agar, 10% MB, 10% Marine Agar (MA)	20-28°C	72 hours	DNA extraction	*Dicentrarchus labrax*
		28°C	48 hours		
Douillet & Holt 1994	Zobell 2216 and TSB + salt (1.5%) and agar	/	1 month	DAPI staining of seawater from culture flask	*Cynoscion nebulosus, Ocyurus chrysurus, Sciaenops ocellatus*
El-Dakour et al. 2015	TCBS, Brain Heart Infusion (BHI) agar	/	/		*Sparidentex hasta*

Escaffre et al. 2001	Pétrifilm Flore totale	21°C	4 days		*Sparus aurata*
Forberg et al. 2011	Liquid and solid M-65 agar, MB	22°C	1 month	Flow cytometer	*Gadus morhua*
Harboe et al. 1994	M-65 agar	10°C	12 days		*Hippoglossus hippoglossus*
Hirazawa et al. 1999	BHI broth +1% NaCl	25°C	24 hours		*Pagrus major, Verasper variegatus*
Katharios et al. 2007	TSA+2% NaCl	20°C	5 days		*Diplodus sargus sargus, Pagrus pagrus*
Kitancharoen et al. 1997	GY (Glucose 2%, Yeast extract 0.5%) agar	20°C	3 days		*Oncorhynchus mykiss*
Kumagai & Nawata 2010	Anacker and Ordal's agar plate for *F. psychrophilum*	15°C	7 days	indirect fluorescent antibody technique	*Oncorhynchus masou, Oncorhynchus kisutch, Oncorhynchus mykiss, Salvelinus pluvius*
Kumagai et al. 1998	Anacker and Ordal's agar plate for *C. psychrophila*	15°C	7 days	indirect fluorescent antibody technique	*Oncorhynchus mykiss*
Lahnsteiner 2017	TSB, Mac Conkey broth, eosin methylene blue broth, Rogosa broth + 10% Tween-80	37°C	18-48 hours		*S. trutta*
Morehead & Hart 2003	TCBS	24°C	64 hours		*Latris lineata*
Overton et al. 2010	Blood agar base (oxoid) with 5% citrated calf blood, MA	20°C	72 hours		*Gadus morhua*
Peck et al. 2004	TSA+1.5% NaCl+ 5% sheep blood	37°C	4 days		*Gadus morhua, Melanogrammus aeglefinus*
Salvesen et al. 1997	M-65 agar	10°C	7 days		*Hippoglossus hippoglossus, Scophthalmus maximus*
Shaw 1957	Difco nutrient broth, Difco nutrient agar, thioglycollate media	/	/		*Tilapia macrocephala*

Situmorang et al. 2014	Lysogeny Broth (LB) and LB agar	27°C	96hours		*Oreochromis niloticus*
Sorensen et al. 2014				SEM quantification of surface coverage	*Anguilla anguilla*
Stuart et al. 2010	Agar (type not mentioned)	/	48 hours		*Atractoscion nobilis, Paralichthys californicus, Seriola lalandi*
Treasurer et al. 2005	MA	10°C	10 days		*Gadus morhua, Melanogrammus aeglefinus*
Verner-Jeffreys et al. 2007	MB, MA	25°C	7 days		*Polydactylus sexfilis, Seriola rivoliana*
Wagner et al. 2010	TSA, enhanced Ordahl's agar with antibiotic (tobramycin)	15°C	12 days		*Oncorhynchus mykiss*
Wagner et al. 2012	Tryptone yeast extract agar (TYES)	15°C	14 days		*Oncorhynchus mykiss*
Wagner et al. 2012	tubes of peptone diluent	4°C	overnight		*Oncorhynchus clarkii, Oncorhynchus mykiss, Salmo trutta*
	followed by plating on tryptone yeast extract agar (TYES)	15°C	14 days		
Wright & Snow 1975	Nutrient Broth	/	/		*Micropterus salmonides*

*Table 3.2 - Overview of studies using hydrogen peroxide as egg disinfectant to reduce either the fungal (F) or bacterial (B) load. Next to the species used, also the concentration and contact time are listed as well as the overall results, relative to the control group, concerning sterility and hatchability. TSA: Tryptic Soy Agar, TCBS: Thiosulfate-citrate-bile salts-sucrose agar. *CT= concentration x contact time*

Order	B/F	Species	Concentration	Contact time (min)	Results			Reference
					Microbial load	Hatchability	Extra information	
Acipenseriformes	F	*Acipenser fulvescens*	0-6000 µl/L	15 (daily)	Fungal contamination on all samples	1000 µl and 3000 µl significantly higher than 6000 µl and control		Rach et al. 1998
	F	*Polyodon spathula*	0-6000 µl/L	15 (daily)	No fungi	1000 µl significantly higher than 3000 µl, 6000 µl and control		Rach et al. 1998
Cypriniformes	F	*Lepidomeda copei*	5000 mg/L	1min or 1min (daily for 5 days)	Daily treatment: no fungi	No significant differences	Test were performed both in petri dishes and in mini jars	Wagner et al. 2012a
	F	*Catostomus commersoni*	0-6000 µl/L	15 (daily)	No fungi	1000 µl and 3000 µl significantly higher than 6000µl and control		Rach et al. 1998
	F	*Cyprinus carpio*	0-6000 µl/L	15 (daily)	No fungi	All treatments significantly higher than control		Rach et al. 1998
Esociformes	F	*Esox lucius*	0-6000 µl/L	15 (daily)	No fungi	No significant differences		Rach et al. 1998
Gadiformes	B	*Gadus morhua*	3%	5	Significant reduction	Significantly higher than control	Significant interaction between treatment	Peck et al. 2004

							and developmental stage	
	B	*Melanogrammus aeglefinus*	3%	5	No sterility	Significantly higher than control		Peck et al. 2004
Perciformes	B	*Sparus aurata*	300 ppm	5 to 20	TSA: bacterial load decrease; TCBS: negative for CT 4500 and 6000	CT 1600 and CT 3200 significantly lower than control		Can et al. 2010
	B	*Pagrus pagrus*	300 ppm	5 to 20	TSA: bacterial load decrease; TCBS: negative for CT 6000	CT 1600 and CT 3200 significantly lower than control		Can et al. 2010
	B	*Dentex dentex*	300 ppm	5 to 20	TSA: bacterial load decrease; TCBS: negative for CT 4500 and 6000	CT 6000 significantly lower than control		Can et al. 2010
	B	*Sciaenops ocellatus*	3%	5	Depending on the trial, liquid and solid culture media: negative in 67% and 83% of the samples	Survival 53 and 69%	Higher concentrations: mortality increased; no longterm effects on survival or growth	Douillet & Holt 1994
	B	*Ocyurus chrysurus*	1%		No sterility		Higher concentrations: mortality increased	Douillet & Holt 1994
	B	*Cynoscion nebulosus*	2%		No sterility		Higher concentrations: mortality increased	Douillet & Holt 1994

	B	*Sparidentex hasta*	50-250 ppm	4	Significant decrease	No significant effect	Significant higher larval survival at 3 days post hatching for 100 and 150 ppm	El-Dakour et al. 2015
	B	*Polydactylus sexfilis*	11340 and 34230 mg/L	5	Decrease	Significantly decreased for 34230 mg/L	Significant batch difference	Verner-Jeffreys et al. 2007
	B	*Seriola rivoliana*	11340 and 34230 mg/L	5	No significant difference	For 34230 mg/L: lethal when eggs were exposed 24h post fertilization, normal hatching when disinfected 3h post fertilization	Significant batch difference	Verner-Jeffreys et al. 2007
	F	*Stizostedion vitreum*	0-6000 µl/L	15 (daily)	No fungi	1000 µl and 3000 µl significantly higher		Rach et al. 1998
	F	*Perca flavescens*	0-6000 µl/L	15 (daily)	No fungi	1000 µl and 3000 µl significantly higher than 6000 µl and control		Rach et al. 1998
Salmoniformes	B	*Oncorhynchus mykiss*	6 g/L	5	Decreased bacterial load	Normal	No deformations	Wagner et al. 2010
	B		30 g/L	1	TSA: in 40 % of the eggs the plates were negative	Normal	No deformations	Wagner et al. 2010
	B		1.50%	2		Normal for all stages except the eyed stage	Different developmental stages	Wagner et al. 2012a

F		100-500 µl	15 (every other day)	500 µl: significant reduction	500 µl: significantly higher than control	Schreier et al. 1996
F		1000 µl	15 (every other day)	Significant reduction	Normal	Schreier et al. 1996
B		15 g/L	2	No effect	Normal	Wagner et al. 2010
F		250 µg/mL, 500 µg/mL and 1000 µg/mL	60 (twice a week)	Significant reduction	Significant increase	Kitancharoen et al. 1997
F		250 mg/L, 500 mg/L and 1000 mg/L	15 (daily)	Significant reduction, most effective for 1000 mg/L	No significant differences	Barnes et al. 1998
B	*Oncorhynchus clarkii lewisii*	1 g/L	15	Decrease in bacterial load	Normal	Wagner et al. 2012a
B		10 g/L	2 & 3	Decrease in bacterial load	Normal	Wagner et al. 2012a
B		15 g/L	2	Decrease in bacterial load	Normal	Wagner et al. 2012a
B		500 mg/L	35 (daily)	Decrease in bacterial load		Wagner et al. 2012a
F	*Oncorhynchus tshawytscha*	500 ppm and 1000 ppm	15 (daily)	Decrease in fungal load	Significantly higher than control	Waterstrat & Marking 1995

Siluriformes	F	*Ictalurus punctatus*	0-6000 µl/L	15 (daily)	No fungi	1000 µl and 3000 µl significantly higher for 6000µl and control	Rach et al. 1998
	F		433 mg/L	daily	Significant reduction	Significant increase	Mitchell et al. 2010
	F		70 mg/L	daily	No fungal growth	Significant increase	Small 2009
		Clarias gariepinus	25 to 2000 ppm	15,30 & 60		Significant increase for ≥ 25 ppm	Rasowo et al. 2007
Perciformes	B	*Oreochromis niloticus*	2% H_2O_2+ Antibiotic mixture: 10 mg/L ampicillin, 10 mg/L rifampicin, 10mg/L trimethoprim, 10 mg/L gentamycin and antifungal products: Amphotericin-B (0.5 mg/L) and Fluorescent Brightener (25 mg/L) + 75mg/L sodium hypochlorite + Antibiotic and antifungal mixture: idem	10 min + 24 h + 2 min + until hatching	Lysogeny broth and plating: ½ negative	Normal	Situmorang et al. 2014

*Table 3.3 - Overview of studies using glutaraldehyde as egg disinfectant to reduce either the fungal (F) or bacterial (B) load. Next to the species used, also the concentration and contact time are listed as well as the overall results, relative to the control group, concerning sterility and hatchability. TSA: Tryptic Soy Agar, TCBS: Thiosulfate-citrate-bile salts-sucrose agar. *CT= concentration x contact time*

Order	B/F	Species	Concentration	Contact time (min)	Results			Reference
					Microbial load	Hatchability	Extra information	
Gadiformes	B	*Gadus morhua*	100 to 800 mg/L	10	Bacterial load decreases	No negative results	Large batch effect	Overton et al. 2010
Salmoniformes	F	*Oncorhynchus mykiss*	25, 50, 100, 200 ppm	60 (daily)	reduction in fungal load for ≥50	Decrease for ≥100 ppm		Cline & Post 1972
	F		No clear indication of tested concentrations	15 or 60	50 ppm or more: no fungus	Decrease for 300 ppm	Toxicity test (also *in vitro*)	Marking et al. 1994
Perciformes	B	*Anarhichas lupus*	600 mg/L	5 (every third day until age 22d and every fith day from day 56 until hatching)	No *Flexibacter* sp.	Decreased during disinfection		Pavlov & Moksness 1993
	B	*Sparus aurata*	200 ppm	2 to 16	TCBS: negative TSA: negative for CT 800, 1600 & 3200	Decrease for CT 3200		Can et al. 2010
	B	*Pagrus pagrus*	100, 200, 400 mg/L	5	Significant reduction for 200 and 400 mg/L	Decrease for protocols with > 100 mg/L		Katharios et al. 2007

	B		200 ppm	2 to 16	TCBS: negative TSA: negative for CT 1600 & 3200	Decrease for CT 3200		Can et al. 2010
	B	*Dentex dentex*	200 ppm	2 to 16	TCBS: negative TSA: negative for CT 1600 & 3200	Decrease for CT 3200		Can et al. 2010
	B	*Latris lineata*	0-3200 ppm	10	TCBS: negative for ≥400 ppm	(Almost) no hatching for 800, 1600 & 3200 ppm		Morehead & Hart 2003
	B	*Diplodus sargus sargus*	100, 200, 400 mg/L	5	Significant reduction for 400 mg/L	Decrease for > 100 mg/L		Katharios et al. 2007
Pleuronectiformes	B	*Hippoglossus hippoglossus*	400 ppm	10	Significant decrease in bacterial load	No differences	Looked for deformations and long term effects on the larvae	Harboe et al. 1994
	B		800 ppm	2.5	Significant decrease in bacterial load	No differences	Looked for deformations and long term effects on the larvae	Harboe et al. 1994
	B		400-1200 mg/L	2.5; 5 & 10	80% of the eggs was sterile in 8/10 with 10 min treatment and only 3 times for 2,5 min treatment	Decrease for 1600 ppm	-Positive effects more pronounced in poor quality batches -Better larval survival when treated for 10	Salvesen et al. 1997

							min. -Stress test	
	B	*Pleuronectes platessa L.*	400, 800, 1600 ppm	10	Significant decrease in bacterial load	No differences	Survival after hatching is lower for ≥800 ppm	Salvesen et al. 1991
	B	*Scopthalmus maximus*	400-1200 mg/L	2.5; 5 & 10	M-65 agar: negative between 95 and 100%	-10 min treatment: very toxic -decrease for ≥ 800 mg/L for 5 min and 1200 mg/L for 2,5 min	Stress test	Salvesen et al. 1997
Perciformes	B	*Dicentrarchus labrax*	100 mg/L	3	Plating and broth: negative	Significantly higher than control		Dierckens et al. 2009
			+ANTIBIOTICS: 10mg/L rifampincin en 10 mg/L ampicillin + first: 0.5% iodine for 10 min (>24h before)		DNA extraction: no bacterial DNA found			
Gadiformes	B	*Gadus morhua*	2x 400 ppm	10	Sterile	Significantly higher than control		Forberg et al. 2011
			+ ANTIBIOTICS: 10 mg/L rifampincin & 10 mg/L ampicillin					
			1x400 ppm	10	No sterility	Significantly higher than control		Forberg et al. 2011

Table 3.4 - Overview of studies using ozone as egg disinfectant to reduce either the fungal (F) or bacterial (B) load. Next to the species used, also the concentration and contact time are listed as well as the overall results, relative to the control group, concerning sterility and hatchability. TSA: Tryptic Soy Agar, TCBS: Thiosulfate-citrate-bile salts-sucrose agar.
**CT= concentration x contact time*

Order	B/F	Species	Concentration	Contact time (min)	Results			Reference
					Microbial load	Hatchability	Extra information	
Gadiformes	B	*Gadus morhua*	0.2 to 10 mg/L	0.5; 1; 3 & 5		Decrease when > 4mg/L for 1 min	No deformations, hatching in well plates	Grotmol et al. 2003
Perciformes	B	*Sparus aurata*	0.5 mg/L	2, 4, 8 & 16	TSA: negative for ≥ CT 2 TCBS: negative for ≥ CT 1	Decrease for CT 8		Can et al. 2012
	B		0.3 mg/L	2, 4, 8 & 16	TSA: negative	Decrease for CT 2,4 and 4,8	Significant more swimbladder inflation	Ben-Atia et al. 2007
	B	*Dicentrarchus labrax*	0.5 mg/L	2, 4, 8 & 16	TSA: negative for ≥ CT 4 TCBS: negative	Decrease for CT 8		Can et al. 2012
	B	*Dentex dentex*	0.5 mg/L	2, 4, 8 & 16	TSA: negative for ≥ CT 2 TCBS: negative	Decrease for CT 8		Can et al. 2012
	B	*Pagrus pagrus*	0.5 mg/L	2, 4, 8 & 16	TSA: negative from CT2 TCBS:	Decrease for CT 8		Can et al. 2012

					negative for ≥ CT1			
	B	*Argyrosomus japonica*	0.1; 0.5; 1 & 5 mg/L	1		Decrease for CT 5	Lower temperature: higher toxicity	Ballagh et al. 2011
	B	*Latris lineata*	0.5; 1, 2 & 5 mg/L	0.5; 1 & 5		3 days old: decrease for CT>2,5 Other stages: decrease for concentration> 2 mg/L	No significant effect of different developmental stages	Battaglene & Morehead 2006
Pleuronectiformes	B	*Scophthalmus maximus*	0.2 to 10 mg/L	0.5; 1; 3 & 5 min		Decrease ≥ 4mg/L for 1 min	No deformations, hatching in well plates	Grotmol et al. 2003
	B		0.2 to 10 mg/L	0.5; 1; 3 & 5 min		Decrease at 4.6 mg/L for 3 to 5 min	High resilience to ozone, no deformations, hatching in well plates	Grotmol et al. 2003
Salmoniformes	F	*Salmo trutta fario*	0.01; 0.03 & 0.2 ppm	10 min daily	Delay of saprolegniasis			Forneris et al. 2003
			0.01; 0.1 & 0.3 ppm	10 min every other day	Delay of saprolegniasis			Forneris et al. 2003

*Table 3.5 - Overview of studies using iodine as egg disinfectant to reduce either the fungal (F) or bacterial (B) load. Next to the species used, also the concentration and contact time are listed as well as the overall results, relative to the control group, concerning sterility and hatchability. MA: Marine Agar, TSA: Tryptic Soy Agar, TCBS: Thiosulfate-citrate-bile salts-sucrose agar. *CT= concentration x contact time*

Order	B/F	Species	Concentration	Contact time (min)	Results			Reference
					Microbial load	Hatchability	Extra information	
Cypriniformes	F	*Cyprinus carpio*	0-200 mg/L	Flush treatment twice a day	Reduction in fungal infection	Slightly better than control, but not very high		Kodabandeh & Abtahi 2006
Gadiformes	B	*Gadus morhua*	10 to 150 mg/L	10	MA and Blood Agar: Decrease in bacterial load	Normal	Large batch effect	Overton et al. 2010
Perciformes	B	*Sparus aurata*	300 ppm	5 to 20	TSA: no effect TCBS: negative	No significant difference		Can et al. 2010
	B	*Pagrus pagrus*	300 ppm	5 to 20	TSA: bacteral load decreased significantly TCBS: negative for CT 4500 & 6000	No significant difference		Can et al. 2010
	B		25, 50, 100 mg/L	5	Significant decrease	Significant decrease for all treatments	0 day old eggs are less tolerant than 1 day old eggs to disinfection	Katharios et al. 2007

B	*Pagrus major*	0- 150 ppm	5	No bacterial growth after 24h for 0.1 & 0.2% for 5 min			Hirazawa et al. 1999
B	*Dentex dentex*	300 ppm	5 to 20	TSA: bacteral load decreased significant TCBS: negative for CT 4500 & 6000	No significant difference		Can et al. 2010
B	*Sparidentex hasta*	25-125 ppm	4	Significant decrease	Significant increase	Significant increase in larval survival at 3 days post hatching	El-Dakour et al. 2015
B	*Seriola lalandi*	50 mg/L	15	Decrease in bacterial load, almost zero	Normal	No long term effects	Stuart et al., 2010
B		100 mg/L	10	Decrease in bacterial load, almost zero	Normal	No long term effects	Stuart et al., 2010
B	*Atractoscion nobilis*	50 mg/L	15	Decrease in bacterial load, almost zero	Significant decrease	No long term effects	Stuart et al., 2010
B		100 mg/L	10	Decrease in bacterial load, almost zero	Significant decrease	No long term effects	Stuart et al., 2010

	B	*Diplodus sargus sargus*	25 mg/L , 50 mg/L, 100 mg/L	5	Significant decrease	Significant decrease for all treatments	0 day old eggs are less tolerant than 1 day old eggs to disinfection	Katharios et al., 2007
	B	*Micropterus salmonides*	100 ppm, 150 ppm, 200 ppm	15	Decrease of *Aeromonas liquefaciens* for 150 ppm and no *A. liquefaciens* for 200 ppm	Significant decrease for 200 ppm		Wright & Snow, 1975
Pleuronectiformes	B	*Paralichthys californicus*	50 mg/L	15	Decrease in bacterial load, almost zero	Very low hatching rate (almost zero)	No long term effects	Stuart et al., 2010
	B		100 mg/L	10	Decrease in bacterial load, almost zero	Very low hatching rate (almost zero)	No long term effects	Stuart et al., 2010
	B	*Verasper variegatus*	0.03% to 0.15%	15	No bacterial growth	decrease in hatching for > 0.075%	No deformations	Hirazawa et al. 1999
Salmoniformes	B	*Oncorhynchus mykiss*	100 ppm, 200 ppm	15	Still *C. psychrophila* present			Kumagai et al. 1996
	F		250 ppm	15, 30, 60	Fungal infection prohibited, not enough to control when infected for >10%	Normal		Marking et al. 1994

	F		1000 ppm	15, 30, 60	Fungal infection controlled and treated	Increased		Marking et al. 1994
	B		2000 mg/L	10	Decrease in bacterial load, TSA: negative for 33% of the eggs	Significantly increased	Different developmental stages tested, no effect	Wagner et al. 2010
	B		100 mg/L	15	Decrease in bacterial load	Significantly increased	Different developmental stages tested, no effect	Wagner et al. 2010
	B		100 mg/L	10	No significant effect	Normal		Wagner et al. 2012a
	B		0.05%-5%	10	No *A. liquefaciens* ≥0.5%	Normal hatching for 0-2.5%, no hatching for ≥ 3%		McFadden 1969
	B	*4 salmonid species*	50 ppm	15	No *F.psychropilum* after disinfection pre-fertilisation			Kumagai & Nawata 2010
	B	*Salmo salar*	5-100 mg/L	30	Significant decrease	Normal	Also tolerance tested with concentrations up till 7500 mg/L. No eggs survived	Chalupnicki et al. 2011

						≥5000 mg/L. All treatments were combined with formalin disinfection 1:750 for 15 minutes 3x/week to prevent fungal growth	
	B	*Salmo trutta*	135 ppm	30, 60	Significant decrease	No significant differences in percentage of embryos developing to the eyed stage	Lahnsteiner 2017
	B	*Oncorhynchus kisutch*	0-1000 ppm	15-120	Still *C. psychrophila* present		Kumagai et al 1996
	B	*Oncorhynchus masou*	100 ppm, 200 ppm	15	Still *C. psychrophila* present		Kumagai et al 1996
	B	*Oncorhynchus tshawytscha*	50 ppm	30	Still F. *psychrophilum*		Loch & Faisal, 2016
	B	*Oncorhynchus tshawytscha*	50 mg/L + 100 mg/L	30 + 10	Still F. *psychrophilum*		Loch & Faisal, 2018
Salmoniformes	B	*Salmo salar*	50 mg + 100 mg	30 + 10	No *A. Salmonicida*		Cipriano et al. 2001

Table 3.6 - Overview of studies using formaldehyde, peracetic acid, tannic acid, bronopol, sodium chloride and copper sulphate as disinfectant to reduce either the fungal (F) or bacterial (B) load. Next to the species used, also the concentration and contact time are listed as well as the overall results, relative to the control group, concerning sterility and hatchability. MA: Marine Agar, TYE: Tryptone Yeast Extract.
**CT= concentration x contact time*

Product	Order	B/F	Species	Concentration	Contact time (min)	Results			Reference
						Sterility	Hatchability	Extra information	
Formaldehyde	Acipenseriformes	F	*Acipenser fulvescens*	1500 µl/L, 4500 µl/L or 7500 µl/L	45 (every other day)	No fungal growth for 4500 µl/L and 6000 µl/L	No hatching for 4500 and 7500 µl/L		Rach et al.1997
	Cypriniformes	F	*Catostomus commersoni*	1500 µl/L, 4500 µl/L or 7500 µl/L	45 (every other day)	No fungal growth for 4500 µl/L and 6000 µl/L	Significant higher for 1500 µl/L and 4500 µl/L		Rach et al. 1997
		F	*Cyprinus carpio*	1500 µl/L, 4500 µl/L or 7500 µl/L	45 (every other day)	No fungal growth for 4500 µl/L and 6000 µl/L	Significant increase		Rach et al. 1997
				0-400 mg/L	Flush treatment twice a day	Reduction in fungal infection with increasing concentration	Hatching increases with increasing concentration; significantly higher for 400 mg/L		Khodabandeh & Abtahi 2006
	Perciformes	F	*Stizostedion vitreum*	1500 µl/L, 4500 µl/L & 7500 µl/L	45 (every other day)	No fungal growth for 4500 µl/L and 6000 µl/L	No significant difference		Rach et al. 1997
		B	*Micropterus salmonides*	500-2000 ppm	15	Still *Aeromonas liquefaciens* present	Decrease from 1500 ppm onwards		Wright & Snow, 1975

	Salmoniformes	F	*Oncorhynchus mykiss*	500 µl, 1000 µl & 1500 µl	15 (every other day)	Reduction in fungal infection with increasing concentration	Increased hatching		Schreier et al. 1996
		F		250 mg/l	60	Significant reduction in fungal growth	>65% but no control group		Bailey & Jeffreys 1989
		F		37.5 ppm, 75 ppm, 150 ppm and 300 ppm	60 (daily)	No fungal growth for 150 ppm and 300 ppm	Reduction in hatching for 150 ppm and 300 ppm		Cline & Post 1972
		F	*Oncorhynchus tshawytscha*	500 ppm and 1000 ppm	15 (daily)	Significant decrease in fungal infected eggs	No significant difference		Waterstrat & Marking 1995
		B		500 mg/L, 1 667 mg/L	15 (daily)	Significant decrease	Significant increase	Initial iodine disinfection with 100 mg/L for 10 min	Barnes et al.2005
	Siluriformes	F	*Ictalurus punctatus*	1500 µl/L, 4500 µl/L & 7500 µl/L	45 (every other day)	No fungal growth for 4500 µl/L and 6000 µl/L	Decrease for 4500 µl/L and 6000 µl/L		Rach et al. 1997
		F		250 mg/L	Daily	Significant reduction	Significant decrease		Mitchell et al. 2010
	Perciformes	B	*Seriola lalandi*	100 mg/L	60	Bacterial load decreases	Normal	No long term effects	Stuart et al. 2010
		B		1000 mg/L	15	Bacterial load decreases	Normal	No long term effects	Stuart et al. 2010

		B	*Atractoscion nobilis*	100 mg/L	60	Bacterial load decreases	Normal	No long term effects	Stuart et al. 2010
		B		1000 mg/L	15	Bacterial load decreases	Normal	No long term effects	Stuart et al. 2010
		B	*Sparidentex hasta*	50-250 ppm	4	Significant reduction	Significant increase	Significant increase in larval survival (3 days post-hatching)	El-Dakour et al. 2015
	Pleuronectiformes	B	*Paralichthys californicus*	100 mg/L	60	Bacterial load decreases	Significant decrease	No long term effects	Stuart et al. 2010
		B		1000 mg/L	15	Bacterial load decreases	Normal	No long term effects	Stuart et al. 2010
Peracetic acid	Gadiformes	B	*Melanogrammus aeglefinus*	Kickstart 1%	1	Decrease in bacterial load	Almost zero		Treasurer et al. 2005
		B	*Gadus morhua*	Perosan 3.5 ml/L	1	TYE: negative	Normal		Brown et al. 2005
		B		Kickstart 1%	1	MA: negative	Normal		Treasurer et al. 2005
	Siluriformes	F	*Ictalurus punctatus*	2.5 to 20 mg/L	twice a day	Significant reduction	Significant higher for 2.5, 5 and 10 mg/L. No hatching for 20 mg/L		Straus et al. 2012
Tannic acid	Salmoniformes	B	*Oncorhynchus mykiss*	0.0002%, 0.002% & 0.02%	5 & 15	Decrease of bacterial load	No significant difference		Wagner et al. 2012b

Bronopol	Gadiformes	B	*Gadus morhua*	0.05%, 0.5% & 1%	0.75	MA: negative for 0.5% and 1%	Increase for 0.05% and 0.5%	No long term effects	Treasurer et al. 2005
	Perciformes	B	*Sparidentex hasta*	50-450 ppm	4	Significant reduction for 250, 350 and 450 ppm	No significant difference	No significant difference in larval survival (3 days post hatching)	El-Dakour et al. 2015
	Salmoniformes	F	*Salmo trutta*	50 mg/l	daily	Significant reduction (almost complete elimination)	Significant increase		Aller-Gancedo & Fregeneda-Grandes 2007
Sodium chloride	Cypriniformes	F	*Cyprinus carpio*	25000, 35000 & 45000 mg/L	Twice a day	Significant reduction	Significant increase		Khodabandeh & Abtahi 2006
	Salmoniformes	F	*Oncorhynchus mykiss*	30000 mg/L	15 (every other day)	Significant decrease	Significant increase		Schreier et al. 1996
		F		30000 ppm	15, 30 or 60	Significant decrease	Significant increase		Marking et al. 1994
		F		15 ppt, 20 ppt & 25 ppt	60 (twice a week)	Significant decrease	Significant increase		Kitancharoen et al. 1997
		F		3 ppt, 5 ppt & 7 ppt	Continous	Significant decrease	Significant increase		Kitancharoen et al. 1997
		F	*Oncorhynchus tshawytscha*	15000 & 30000 ppm	15 (daily)	Significant decrease for 30000 ppm	No significant effect		Waterstrat & Marking 1995
Copper sulphate	Cypriniformes	F	*Lepidomeda copei*	40 mg/L, 60 mg/L	2	No fungi	Significant increase		Wagner et al. 2012a

	Perciformes	F	*Morone chrysops x M. saxatilis*	10 to 40 mg/L	10 (3 times)	Maximal fungal reduction at 20 mg/L	Significant increase at 20 and 40 mg/L	LC50 for larvae: 5.4 mg/L at 24 hours, 3.9 mg/L at 48 hours	Strauss et al. 2016
	Salmoniformes	B	*Oncorhynchus mykiss*	100, 300, 500 & 700 mg/L	15	Reduction for ≥300 mg/L	Significant decrease at 500 and 700 mg/L		Wagner & Oplinger, 2013
		F	*Ictalurus punctatus*	2.5 to 40 mg/L	daily	Significant decrease for all treatments	Significant increase for all treatments		Straus et al. 2009b
				10 mg/L	daily	Significant decrease	Significant increase		Mitchell et al. 2010

3.7 REFERENCES

Abd El-Gawad, E.A., Shen, Z.G. & Wang, H.P. (2016) Efficacy of formalin, iodine and sodium chloride in improvement of egg hatching rate and fry survival prior to the onset of exogenous feeding in yellow perch. *Aquaculture Research,* **47,** 2461-2469.

Aerts, J., Schaeck, M., De Swaef, E., Ampe, B. & Decostere, A. (2018) Vibrio lentus as a probiotic candidate lowers glucocorticoid levels in gnotobiotic sea bass larvae. *Aquaculture,* **492,** 40-45.

Agency, U.S.E.P. (1995) R.E.D. Facts Bronopol. In: *Prevention, Pesticides and Toxic Substances (7508W)*.

Alderman, D.J. (1984) The Toxicity of Iodophors to Salmonid Eggs. *Aquaculture,* **40,** 7-16.

Aller-Gancedo, J.M. & Fregeneda-Grandes, J.M. (2007) Comparative efficacy of Pyceze (R) (bronopol) in controlling mortality of brown trout *Salmo trutta* eggs. (vol 38, pg 618, 2007). *Aquaculture Research,* **38,** 891-891.

Alsop, D. & Wood, C.M. (2011) Metal uptake and acute toxicity in zebrafish: Common mechanisms across multiple metals. *Aquatic Toxicology,* **105,** 385-393.

Amend, D.F. (1974) Comparative Toxicity of 2 Iodophors to Rainbow-Trout Eggs. *Transactions of the American Fisheries Society,* **103,** 73-78.

Anbalagan, M., Ganesh Prabu, P., Krishnaveni, R.E. & Manivannan, S. (2013) Effect of sodium chloride (nacl) on the bacterial load in chicken, mutton and beef meat samples in relation to meat spoilage. *International Journal of Research studies in Zoology,* **4**, 1-5.

Andrews, J. & Harris, R. (1986) r- and K-Selection and Microbial Ecology. In: *Advances in Microbial Ecology* (ed. by K.C. Marshall), pp. 99-147. Springer US.

Arimoto, M., Sato, J., Maruyama, K., Mimura, G. & Furusawa, I. (1996) Effect of chemical and physical treatments on the inactivation of striped jack nervous necrosis virus (SJNNV). *Aquaculture,* **143,** 15-22.

Aydın, I., Polat, H., Şahin, T., Kolotoğlu, L. & Küçük, E. (2011) Effect of iodine treatment on the hatching rate of Black Sea Turbot (*Psetta maxima* Linnaeus, 1758), eggs. *Journal of Fisheries Sciences,* **5,** 73-78.

Bailey, T.A. & Jeffrey, S.M. (1989) Evaluation of 215 candidate fungicides for use in fish culture. In: *Investigations in Fish Control*. US Fish and Wildlife Service.

Bakke, I., De Schryver, P., Boon, N., Vadstein, O. (2011) PCR-based community structure studies of Bacteria associated with eukaryotic organisms: A simple PCR strategy to avoid co-amplification of eukaryotic DNA. *Journal of Microbiological Methods,* **84**, 349-351.

Ballagh, D.A., Pankhurst, P.M. & Fielder, D.S. (2011) Embryonic development of mulloway, *Argyrosomus japonicus*, and egg surface disinfection using ozone. *Aquaculture,* **318,** 475-478.

Barnes, M.E., Bergmann, D., Jacobs, J. & Gabel, M. (2009) Effect of *Flavobacterium columnare* inoculation, antibiotic treatments and resident bacteria on rainbow trout *Oncorhynchus mykiss* eyed egg survival and external membrane structure. *Journal of Fish Biology,* **74,** 576-590.

Barnes, M.E., Bergmann, D., Stephenson, H., Gabel, M. & Cordes, R.J. (2005) Bacterial numbers from landlocked fall Chinook salmon eyed eggs subjected to various formalin treatments as determined by scanning electron microscopy and bacteriological culture methods. *North American Journal of Aquaculture,* **67,** 23-33.

Barnes, M.E., Cordes, R.J., Sayler, W.A. & Hanten, R.P. (2003) Soft-egg disease in landlocked fall chinook salmon eggs: Possible causes and therapeutic treatments. *North American Journal of Aquaculture,* **65,** 126-133.

Barnes, M.E., Ewing, D.E., Cordes, R.J. & Young, G.L. (1998) Observations on hydrogen peroxide control of *Saprolegnia* spp. during rainbow trout egg incubation. *Progressive Fish-Culturist,* **60,** 67-70.

Barnes, M.E., Sayler, W.A. & Cordes, R.J. (2001) Use of formalin treatments during incubation of eyed eggs of brown trout. *North American Journal of Aquaculture,* **63,** 333-337.

Barnes, M.E., Stephenson, H. & Gabel, M. (2003) Use of hydrogen peroxide and formalin treatments during incubation of landlocked fall chinook salmon eyed eggs. *North American Journal of Aquaculture,* **65,** 151-154.

Barnes, M.E., Wintersteen, K., Sayler, W.A. & Cordes, R.J. (2000) Use of formalin during incubation of rainbow trout eyed eggs. *North American Journal of Aquaculture,* **62,** 54-59.

Battaglene, S.C. & Morehead, D.T. (2006) Tolerance of striped trumpeter *Latris lineata* embryos to ozonated seawater. *Aquaculture International,* **14,** 421-429.

Ben-Atia, I., Lutzky, S., Barr, Y., Gamsiz, K., Shtupler, Y., Tandler, A. & Koven, W. (2007) Improved performance of gilthead sea bream, *Sparus aurata*, larvae after ozone disinfection of the eggs. *Aquaculture Research,* **38,** 166-173.

Bergh, Ø. & Jelmert, A. (1990) Antibacterial treatment procedures of eggs of halibut (*Hippoglossus hippoglossus* L.). In: *Council meeting. International Council for the Exploration of the sea/F:39*.

Birkbeck, T.H., Reid, H.I., Darde, B. & Grant, A.N. (2006) Activity of bronopol (Pyceze((R))) against bacteria cultured from eggs of halibut, *Hippoglossus hippoglossus* and cod, *Gadus morhua*. *Aquaculture,* **254,** 125-128.

Blancheton, J.P., Attramadal, K.J.K., Michaud, L., D'orbcastel, E.R. & Vadstein, O. (2013) Insight into bacterial population in aquaculture systems and its implication. *Aquacultural Engineering,* **53,** 30-39.

Block, S.S. (2001) *Disinfection, sterilization, and preservation /Ed. Seymour S. Block,* Lippincott Williams and Wilkins.

Bolker, J.A. & Hill, C.R. (2000) Pigmentation development in hatchery-reared flatfishes. *Journal of Fish Biology,* **56,** 1029-1052.

Bouchard, H.J. & Aloisi, D.B. (2002) Investigations in concurrent disinfection and de-adhesion of lake sturgeon eggs. *North American Journal of Aquaculture,* **64,** 212-216.

Brown, L.L., Cox, W.T. & Levine, R.P. (1997) Evidence that the causal agent of bacterial cold-water disease *Flavobacterium psychrophilum* is transmitted within salmonid eggs. *Diseases of Aquatic Organisms,* **29,** 213-218.

Brown, N., Kling, L. & Skonberg, D. (2005) Final report to NOAA: Demonstration of Sustainable Cod Farming from Egg to Grow-out in Maine. In, p. 55. University of Maine.

Bruno, D.W. & Munro, A.L.S. (1986) Observations on *Renibacterium Salmoninarum* and the Salmonid Egg. *Diseases of Aquatic Organisms,* **1,** 83-87.

Buchan, K.a.H., Martin-Robichaud, D.J., Benfey, T.J., Mackinnon, A.M. & Boston, L. (2006) The efficacy of ozonated seawater for surface disinfection of haddock (*Melanogrammus aeglefinus*) eggs against piscine nodavirus. *Aquacultural Engineering,* **35,** 102-107.

Can, E., Karacalar, U., Saka, S. & Firat, K. (2012) Ozone Disinfection of Eggs from Gilthead Seabream *Sparus aurata*, Sea Bass *Dicentrarchus labrax*, Red Porgy *Pagrus pagrus*, and Common Dentex *Dentex dentex*. *Journal of Aquatic Animal Health,* **24,** 129-133.

Can, E., Saka, S. & Firat, K. (2010) Disinfection of Gilthead Sea Bream (*Sparus aurata*), Red Porgy (*Pagrus pagrus*), and Common Dentex (*Dentex dentex*) Eggs from Sparidae with Different Disinfectants. *Kafkas Universitesi Veteriner Fakultesi Dergisi,* **16,** 299-306.

Caruffo, M., Nayarrete, N.C., Salgado, O.A., Faundez, N.B., Gajardo, M.C., Feijoo, C.G., Reyes-Jara, A., Garcia, K. & Navarrete, P. (2016) Protective Yeasts Control *V. anguillarum* Pathogenicity and Modulate

the Innate Immune Response of Challenged Zebrafish (*Danio rerio*) Larvae. *Frontiers in Cellular and Infection Microbiology,* **6**.

CFR (2013) Code of Federal Regulations Title 21. ed. by U.S.F.A.D. Administration.

Chalupnicki, M.A., Ketola, H.G., Starliper, C.E. & Gallagher, D. (2011) Efficacy and Toxicity of Iodine Disinfection of Atlantic Salmon Eggs. *North American Journal of Aquaculture,* **73,** 124-128.

Chambel, J., Costa, R., Gomes, M., Mendes, S., Baptista, T. & Pedrosa, R. (2014) Hydrogen peroxide, iodine solution and methylene solution highly enhance the hatching rate of freshwater ornamental fish species. *Aquaculture International,* **22,** 1743-1751.

Chan-Yeung, M., Mcmurren, T., Catoniobegley, F. & Lam, S. (1993) Occupational Asthma in a Technologist Exposed to Glutaraldehyde. *Journal of Allergy and Clinical Immunology,* **91,** 974-978.

Chatain, B. (1987) The Swimbladder in *Dicentrarchus Labrax* and *Sparus Auratus* - Influence of Development Anomalies on Larval Growth. *Aquaculture,* **65,** 175-181.

Chung, S.K., Ng, A., Min, H.Y., Strattonthomas, J., Rosenberg, S., Shuman, M. & Hwang, D.G. (1995) Inhibition of Basic Fibroblast Growth Factor-Induced Corneal Angiogenesis by a Urokinase Plasminogen-Activator Receptor Antagonist. *Investigative Ophthalmology & Visual Science,* **36,** S30-S30.

Cipriano, R.C., Novak, B.M., Flint, D.E. & Cutting, D.C. (2001) Reappraisal of the federal fish health recommendation for disinfecting eggs of Atlantic salmon in iodophor. *Journal of Aquatic Animal Health,* **13,** 320-327.

Cline, T.F. & Post, G. (1972) Therapy for Trout Eggs Infected with *Saprolegnia*. *Progressive Fish-Culturist,* **34,** 148-&.

Colwell, R.R. & Grimes, D.J. (1984) Vibrio Diseases of Marine Fish Populations. *Helgolander Meeresuntersuchungen,* **37,** 265-287.

Cornwell, E.R., Groocock, G.H., Getchell, R.G. & Bowser, P.R. (2011) Residual Tannic Acid Destroys Virucidal Properties of Iodophor. *North American Journal of Aquaculture,* **73,** 8-12.

Cullinan, P., Hayes, J., Cannon, J., Madan, I., Heap, D. & Taylor, A.N. (1992) Occupational Asthma in Radiographers. *Lancet,* **340,** 1477-1477.

Dabrowski, K., Ware, K., Jaroszewska, M. & Kwasek, K. (2009) Evaluation of Walleye Embryo Survival and Larval Viability after Iodine Treatment. *North American Journal of Aquaculture,* **71,** 122-129.

Denyer, S.P. & Stewart, G.S.a.B. (1998) Mechanisms of action of disinfectants. *International Biodeterioration & Biodegradation,* **41,** 261-268.

Dierckens, K., Rekecki, A., Laureau, S., Sorgeloos, P., Boon, N., Van Den Broeck, W. & Bossier, P. (2009) Development of a bacterial challenge test for gnotobiotic sea bass (*Dicentrarchus labrax*) larvae. *Environmental Microbiology,* **11,** 526-533.

Douillet, P.A. & Holt, G.J. (1994) Surface Disinfection of Red Drum (*Sciaenops Ocellatus* Linnaeus) Eggs Leading to Bacteria-Free Larvae. *Journal of Experimental Marine Biology and Ecology,* **179,** 253-266.

Edgell, P., Lawseth, D., Mclean, W.E. & Britton, E.W. (1993) The Use of Salt-Solutions to Control Fungus (*Saprolegnia*) Infestations on Salmon Eggs. *Progressive Fish-Culturist,* **55,** 48-52.

Ekman, E., Borjeson, H. & Johansson, N. (1999) *Flavobacterium psychrophilum* in Baltic salmon *Salmo salar* brood fish and their offspring. *Diseases of Aquatic Organisms,* **37,** 159-163.

El-Dakour, S., Saheb, A.I. & Al-Abdul-Elah, K. (2015) Effects of commonly used disinfectants on bacterial load, hatchability and survival of Bluefin Sea bream (*Sparidentex hasta*) eggs. *Aquaculture Research,* **46,** 1281-1291.

Escaffre, A.M., Bazin, D. & Bergot, P. (2001) Disinfection of *Sparus aurata* eggs with glutaraldehyde. *Aquaculture International,* **9,** 451-458.

Evelyn, T.P.T., Ketcheson, J.E. & Prosperiporta, L. (1984) Further Evidence for the Presence of *Renibacterium Salmoninarum* in Salmonid Eggs and for the Failure of Povidone-Iodine to Reduce the Intra-Ovum Infection-Rate in Water-Hardened Eggs. *Journal of Fish Diseases,* **7,** 173-182.

Evelyn, T.P.T., Ketcheson, J.E. & Prosperiporta, L. (1986) Use of Erythromycin as a Means of Preventing Vertical Transmission of *Renibacterium Salmoninarum*. *Diseases of Aquatic Organisms,* **2,** 7-11.

Evelyn, T.P.T., Prosperiporta, L. & Ketcheson, J.E. (1986) Experimental Intraovum Infection of Salmonid Eggs with *Renibacterium Salmoninarum* and Vertical Transmission of the Pathogen with Such Eggs Despite Their Treatment with Erythromycin. *Diseases of Aquatic Organisms,* **1,** 197-202.

Evelyn, T.P.T., Prosperiporta, L. & Ketcheson, J.E. (1986) Persistence of the Kidney-Disease Bacterium, *Renibacterium Salmoninarum*, in Coho Salmon, *Oncorhynchus Kisutch* (Walbaum), Eggs Treated during and after Water-Hardening with Povidone-Iodine. *Journal of Fish Diseases,* **9,** 461-464.

Fao (2012) The State of World Fisheries and Aquaculture 2012. In, p. 209, Rome.

Forberg, T., Arukwe, A. & Vadstein, O. (2011) A protocol and cultivation system for gnotobiotic Atlantic cod larvae (*Gadus morhua* L.) as a tool to study host microbe interactions. *Aquaculture,* **315,** 222-227.

Forneris, G., Bellardi, S., Palmegiano, G.B., Saroglia, M., Sicuro, B., Gasco, L. & Zoccarato, I. (2003) The use of ozone in trout hatchery to reduce saprolegniasis incidence. *Aquaculture,* **221,** 157-166.

Fraenkel-Conrat, H., Cooper, M. & Olcott, H.S. (1945) The reaction of formaldehyde with proteins. *Journal of the American Chemical Society,* **67,** 950-954.

Fraise, A.P., Lambert, P.A. & Maillard, J.-Y. (2004) *Russell, Hugo and Ayliffe's principles and practice of disinfection, preservation and sterilisation /ed. by Adam P. Fraise, Peter A. Lambert, Jean-Yves Maillard,* Blackwell.

Froelich, S.L., Engelhardt, T. (1996) Comparative effects of formalin and salt treatments on hatch rate of koi carp eggs. *The Progressive Fish-Culturist,* **58**, 209-211.

Fux, C.A., Shirtliff, M., Stoodley, P. & Costerton, J.W. (2005) Can laboratory reference strains mirror 'real-world' pathogenesis? *Trends in Microbiology,* **13,** 58-63.

Gannon, P.F., Bright, P., Campbell, M., O'hickey, S.P. & Burge, P.S. (1995) Occupational asthma due to glutaraldehyde and formaldehyde in endoscopy and x ray departments. *Thorax,* **50,** 156-159.

Gardner, J.F. & Peel, M.M. (1986) *Introduction to sterilization and disinfection,* Churchill Livingstone

Gee, L.L. & Sarles, W. (1942) The disinfection of trout eggs contaminated with *Bacterium salmonicida*. *Journal of bacteriology,* **44,** 111.

Gottardi, W. (1985) The Influence of the Chemical Behavior of Iodine on the Germicidal Action of Disinfectant Solutions Containing Iodine. *Journal of Hospital Infection,* **6,** 1-11.

Grotmol, S., Dahl-Paulsen, E. & Totland, G.K. (2003) Hatchability of eggs from Atlantic cod, turbot and Atlantic halibut after disinfection with ozonated seawater. *Aquaculture,* **221,** 245-254.

Grotmol, S. & Totland, G.K. (2000) Surface disinfection of Atlantic halibut *Hippoglossus hippoglossus* eggs with ozonated sea-water inactivates nodavirus and increases survival of the larvae. *Diseases of Aquatic Organisms,* **39,** 89-96.

Hansen, G.H. & Olafsen, J.A. (1989) Bacterial-Colonization of Cod (*Gadus Morhua* L) and Halibut (*Hippoglossus Hippoglossus*) Eggs in Marine Aquaculture. *Applied and Environmental Microbiology,* **55,** 1435-1446.

Hansen, G.H. & Olafsen, J.A. (1999) Bacterial interactions in early life stages of marine cold water fish. *Microbial Ecology,* **38,** 1-26.

Hansen, T.K. & Falk-Petersen, I.B. (2001) Effects of egg disinfection and incubation temperature on early life stages of spotted wolffish. *Aquaculture International,* **9,** 333-344.

Harboe, T., Huse, I. & Oie, G. (1994) Effects of Egg Disinfection on Yolk-Sac and 1st Feeding Stages of Halibut (*Hippoglossus Hippoglossus* L) Larvae. *Aquaculture,* **119,** 157-165.

Heydarnejad, M.S. (2012) Survival and growth of common carp (*Cyprinus carpio* L.) exposed to different water pH levels. *Turkish Journal of Veterinary & Animal Sciences,* **36,** 245-249.

Hiney, M.P. & Smith, P.R. (1998) Validation of Polymerase Chain Reaction-based techniques for proxy detection of bacterial fish pathogens: Framework, problems and possible solutions for environmental applications. *Aquaculture,* **162,** 41-68.

Hirazawa, N., Hara, T., Mitsuboshi, T., Okazaki, J. & Hata, K. (1999) Iodophor disinfection of eggs of spotted halibut *Verasper variegatus* and red sea bream *Pagrus major*. *Fisheries Science,* **65,** 333-338.

Hirazawa, N., Hara, T., Okazaki, J. & Hata, K. (1999) Changes of sensitivity of red sea bream *Pagrus major* eggs to iodophor during the spawning season. *Fisheries Science,* **65,** 484-485.

Howarth, R.S. & Sprague, J.B. (1978) Copper Lethality to Rainbow Trout in Waters of Various Hardness and Ph. *Water Research,* **12,** 455-462.

IHCP (2006) European Union Risk Assessment Report: Phenol. In: *Office for Official Publications of the European Communities* (ed. by K.A. S.J. Munn, O. Cosgrove, S. Pakalin, A. Paya-Perez, B. Schwarz-Schulz, & S. Vegro), p. 229. Federal Institute for Occupational Safety and Health Notification Unit, Dortmund, Germany.

Jodun, W.A. & Millard, M.J. (2001) Effect of iodophor concentration and duration of exposure during water hardening on survival of Atlantic salmon eggs. *North American Journal of Aquaculture,* **63,** 229-233.

Johnson, I.D. (2010) *The Molecular Probes Handbook: A Guide to Fluorescent Probes and Labeling Technologies,* 11th Edition, Life Technologies Corporation.

Karo, O., Wahl, A., Nicol, S.B., Brachert, J., Lambrecht, B., Spengler, H.P., Nauwelaers, F., Schmidt, M., Schneider, C.K., Muller, T.H. & Montag, T. (2008) Bacteria detection by flow cytometry. *Clinical Chemistry and Laboratory Medicine,* **46,** 947-953.

Katharios, P., Agathaggelou, A., Paraskevopoulos, S. & Mylonas, C.C. (2007) Comparison of iodine and glutaraldehyde as surface disinfectants for red porgy (*Pagrus pagrus*) and white sea bream (*Diplodus sargus sargus*) eggs. *Aquaculture Research,* **38,** 527-536.

Khodabandeh, S. & Abtahi, B. (2006) Effects of sodium chloride, formalin and iodine on the hatching success of common carp, *Cyprinus carpio*, eggs. *Journal of Applied Ichthyology,* **22,** 54-56.

Kiemer, M.C.B. & Black, K.D. (1997) The effects of hydrogen peroxide on the gill tissues of Atlantic salmon, *Salmo salar* L. *Aquaculture,* **153,** 181-189.

Kitancharoen, N., Ono, A., Yamamoto, A. & Hatai, K. (1997) The fungistatic effect of NaCl on rainbow trout egg Saprolegniasis. *Fish Pathology,* **32,** 159-162.

Kitancharoen, N., Yamamoto, A., Hatai, K. (1997a) Fungicidal effect of hydrogen peroxide on fungal infection of rainbow trout eggs. *Mycoscience,* **38**, 375-378.

Kitis, M. (2004) Disinfection of wastewater with peracetic acid: a review. *Environment International,* **30,** 47-55.

Kumagai, A. & Nawata, A. (2010) Prevention of *Flavobacterium psychrophilum* Vertical Transmission by Iodophor Treatment of Unfertilized Eggs in Salmonids. *Fish Pathology,* **45,** 164-168.

Kumagai, A., Takahashi, K., Yamaoka, S. & Wakabayashi, H. (1998) Ineffectiveness of iodophore treatment in disinfecting salmonid eggs carrying *Cytophaga psychrophila*. *Fish Pathology,* **33,** 123-128.

Lahnsteiner, F. & Kletzl, M. (2015) Iodophor disinfection of non-hardened *Lota lota*, *Salmo trutta*, and *Thymallus thymallus* eggs: Tolerance levels and iodine permeability. *Aquaculture,* **436,** 167-171.

Leung, H.W. (2001) Ecotoxicology of glutaraldehyde: Review of environmental fate and effects studies. *Ecotoxicology and Environmental Safety,* **49,** 26-39.

Li, X., Defoirdt, T., Yang, Q., Laureau, S., Bossier, P. & Dierckens, K. (2014) Host-induced increase in larval sea bass mortality in a gnotobiotic challenge test with *Vibrio anguillarum*. *Diseases of Aquatic Organisms,* **108,** 211-216.

Li, X., Yang, Q., Dierckens, K., Milton, D.L. & Defoirdt, T. (2014) RpoS and Indole Signaling Control the Virulence of *Vibrio anguillarum* towards Gnotobiotic Sea Bass (*Dicentrarchus labrax*) Larvae. *Plos One,* **9**.

Liltved, H., Hektoen, H. & Efraimsen, H. (1995) Inactivation of Bacterial and Viral Fish Pathogens by Ozonation or UV Irradiation in Water of Different Salinity. *Aquacultural Engineering,* **14,** 107-122.

Loch, T.P. & Faisal, M. (2016) Gamete-associated flavobacteria of the oviparous Chinook salmon (*Oncorhynchus tshawytscha*) in lakes Michigan and Huron, North America. *Journal of Microbiology,* **54,** 477-486.

Loch, T.P. & Faisal, M. (2018) Flavobacteria colonizing the early life stages of hatchery-incubated Chinook salmon *Oncorhynchus tshawytscha* (Walbaum 1792) are markedly diverse. *Journal of Fish Diseases,* **41,** 829-845.

Luna, G.M., Manini, E. & Danovaro, R. (2002) Large fraction of dead and inactive bacteria in coastal marine sediments: Comparison of protocols for determination and ecological significance. *Applied and Environmental Microbiology,* **68,** 3509-3513.

Marchand, P.A., Phan, T.M., Straus, D.L., Farmer, B.D., Stuber, A. & Meinelt, T. (2012) Reduction of in vitro growth in *Flavobacterium columnare* and *Saprolegnia parasitica* by products containing peracetic acid. *Aquaculture Research,* **43,** 1861-1866.

Marking, L.L., Rach, J.J. & Schreier, T.M. (1994) Evaluation of Antifungal Agents for Fish Culture. *Progressive Fish-Culturist,* **56,** 225-231.

Marques, A., Ollevier, F., Verstraete, W., Sorgeloos, P. & Bossier, P. (2006) Gnotobiotically grown aquatic animals: opportunities to investigate host-microbe interactions. *Journal of Applied Microbiology,* **100,** 903-918.

Mcdonnell, G. & Russell, A.D. (1999) Antiseptics and disinfectants: Activity, action, and resistance. *Clinical Microbiology Reviews,* **12,** 147-+.

Mcfadden, T.W. (1969) Effective Disinfection of Trout Eggs to Prevent Egg Transmission of *Aeromonas Liquefaciens*. *Journal of the Fisheries Research Board of Canada,* **26,** 2311-&.

Mendicino, L., Science, E.S.D., Division, T. & Meeting, E.S. (2001) *Environmental Issues with Materials and Processes for the Electronics and Semiconductor Industries: Proceedings of the Fourth International Symposium,* Electrochemical Society.

Mimura, G., Katayama, Y., Ji, X., Xie, J. & Namba, K. (1998) Acute toxicity of ozone-exposed seawater and chlorinated seawater for Japanese flounder, *Paralichthys olivaceus*, eggs, larvae and juveniles. *Suisan Zoshoku,* **46,** 569-578.

Mitchell, A.J., Straus, D.L., Farmer, B. & Carter, R. (2010) Comparison of Percent Hatch and Fungal Infestation in Channel Catfish Eggs after Copper Sulfate, Diquat Bromide, Formalin, and Hydrogen Peroxide Treatment. *North American Journal of Aquaculture,* **72,** 201-206.

Mohr, H., Lambrecht, B., Bayer, A., Spengler, H.P., Nicol, S.B., Montag, T. & Muller, T.H. (2006) Basics of flow cytometry-based sterility testing of platelet concentrates. *Transfusion,* **46,** 41-49.

Morehead, D.T. & Hart, P.R. (2003) Disinfection of striped trumpeter (*Latris lineata*) eggs with glutaraldehyde. *Aquaculture International,* **11,** 255-260.

Morrison, C., Bird, C., O'neil, D., Leggiadro, C., Martin-Robichaud, D., Rommens, M. & Waiwood, K. (1999) Structure of the egg envelope of the haddock, *Melanogrammus aeglefinus*, and effects of microbial colonization during incubation. *Canadian Journal of Zoology-Revue Canadienne De Zoologie,* **77,** 890-901.

Munro, P.D., Barbour, A. & Birkbeck, T.H. (1995) Comparison of the Growth and Survival of Larval Turbot in the Absence of Culturable Bacteria with Those in the Presence of *Vibrio Anguillarum, Vibrio Alginolyticus*, or a Marine *Aeromonas* Sp. *Applied and Environmental Microbiology,* **61,** 4425-4428.

Nikolakakis, S., Dierckens, K., Bossier, P. & Adriaens, D. (2018) Phenotypic effects of antibiotic-induced axenity and egg disinfection in early larval European seabass (*Dicentrarchus labrax* L.). *Aquaculture Research,* **49,** 2536-2552.

Novakov, N., Mandic, V., Kartalovic, B., Vidovic, B., Stojanac, N., Kovacevic, Z. & Plavsa, N. (2018) Comparison of the Efficacy of Hydrogen Peroxide and Salt for Control of Fungal Infections on Brown Trout (*Salmo trutta*) Eggs. *Acta Scientiae Veterinariae,* **46**.

Oplinger, R., Wagner, E.J. & Cavender, A. (2015) Use of Penicillin and Streptomycin to Reduce Spread of Bacterial Coldwater Disease II: Efficacy of Using Antibiotics in Diluents and During Water Hardening. *Journal of Aquatic Animal Health,* **27,** 32-37.

Overton, J.L., Bruun, M.S. & Dalsgaard, I. (2010) Chemical surface disinfection of eggs of Baltic cod, *Gadus morhua* L. *Journal of Fish Diseases,* **33,** 707-716.

Oyarbide, U., Iturria, I., Rainieri, S. & Pardo, M.A. (2015) Use of Gnotobiotic Zebrafish to Study *Vibrio anguillarum* Pathogenicity. *Zebrafish,* **12,** 71-80.

Partridge, G.J., Ginbey, B.M., Woolley, L.D., Fairclough, D.V., Crisafulli, B., Chaplin, J., Prokop, N., Dias, J., Bertram, A. & Jenkins, G.I. (2017) Development of techniques for the collection and culture of wild-caught fertilised snapper (*Chrysophrys auratus*) eggs for stock enhancement purposes. *Fisheries Research,* **186,** 524-530.

Pavlov, D. & Moksness, E. (1993) Bacterial destruction of the egg shell of common wolffish during incubation. *Aquaculture International,* **1,** 178-186.

Peck, M.A., Buckley, L.J., O'bryan, L.M., Davies, E.J. & Lapolla, A.E. (2004) Efficacy of egg surface disinfectants in captive spawning Atlantic cod *Gadus morhua* L. and haddock *Melanogrammus aeglefinus* L. *Aquaculture Research,* **35,** 992-996.

Peeler, E.J., Oidtmann, B.C., Midtlyng, P.J., Miossec, L. & Gozlan, R.E. (2011) Non-native aquatic animals introductions have driven disease emergence in Europe. *Biological Invasions,* **13,** 1291-1303.

Perez-Ramos, A., Mohedano, M.L., Pardo, M.A. & Lopez, P. (2018) beta-Glucan-Producing *Pediococcus parvulus* 2.6: Test of Probiotic and Immunomodulatory Properties in Zebrafish Models. *Frontiers in Microbiology,* **9**.

Pham, L.N., Kanther, M., Semova, I. & Rawls, J.F. (2008) Methods for generating and colonizing gnotobiotic zebrafish. *Nature Protocols,* **3,** 1862-1875.

Piper, R.G., McElwain, I.B., Orme, L.E., McCraren, J.P., Fowler, L.G. & Leonard, J.R. (1982) *Fish hatchery management*. Department of the Interior U.S. Fish and Wildlife Service, Washington D. C.

Planas, M. & Cunha, I. (1999) Larviculture of marine fish: problems and perspectives. *Aquaculture,* **177,** 171-190.

Pravecek, J. & Barnes, M.E. (2003) Lack of effect of iodophor on survival of westslope cutthroat trout eggs during water hardening. *North American Journal of Aquaculture,* **65,** 266-268.

Rach, J.J., Gaikowski, M.P., Howe, G.E. & Schreier, T.M. (1998) Evaluation of the toxicity and efficacy of hydrogen peroxide treatments on eggs of warm and cool water fishes. *Aquaculture,* **165,** 11-25.

Rach, J.J., Howe, G.E. & Schreier, T.M. (1997) Safety of formalin treatments on warm- and coolwater fish eggs. *Aquaculture,* **149,** 183-191.

Rach, J.J., Marks, J.A. & Dawson, V.K. (1995) Effect of Water-Flow Rates in Hatching Jars to Control Fungal Infections of Rainbow Trout Eggs. *Progressive Fish-Culturist,* **57,** 226-230.

Rasowo, J., Okoth, O.E. & Ngugi, C.C. (2007) Effects of formaldehyde, sodium chloride, potassium permanganate and hydrogen peroxide on hatch rate of African catfish *Clarias gariepinus* eggs. *Aquaculture,* **269,** 271-277.

Rawls, J.F., Samuel, B.S. & Gordon, J.I. (2004) Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proceedings of the National Academy of Sciences of the United States of America,* **101,** 4596-4601.

Rekecki, A., Dierckens, K., Laureau , S., Boon, N., Bossier, P. & Van den Broeck, W. (2009) Effect of germ-free rearing environment on gut development of larval sea bass (*Dicentrarchus labrax* L.). *Aquaculture,* **293**, 8-15.Rekecki, A., Gunasekara, R.A.Y.S.A., Dierckens, K., Laureau, S., Boon, N., Favoreel, H., Cornelissen, M., Sorgeloos, P., Ducatelle, R., Bossier, P. & Van den Broeck, W. (2012)

Bacterial host interaction of GFP-labelled *Vibrio anguillarum* HI610 with gnotobiotic sea bass (*Dicentrarchus labrax* L.) larvae. *Journal of Fish Diseases*, **35**, 265-273.

Rekecki, A., Ringø, E., Olsen, R., Myklebust, R., Dierckens, K., Bergh, Ø., Laureau, S., Cornelissen, M., Ducatelle, R., Decostere, A., Bossier, P. & Van den Broeck, W. (2013) Luminal uptake of *Vibrio (Listonella) anguillarum* by shed enterocytes – a novel early defence strategy in larval fish. *Journal of Fish Diseases*, **36**, 419–426.

Reiser, S., Schroeder, J.P., Wuertz, S., Kloas, W. & Hanel, R. (2010) Histological and physiological alterations in juvenile turbot (*Psetta maxima*, L.) exposed to sublethal concentrations of ozone-produced oxidants in ozonated seawater. *Aquaculture,* **307,** 157-164.

Reiser, S., Wuertz, S., Schroeder, J.P., Kloas, W. & Hanel, R. (2011) Risks of seawater ozonation in recirculation aquaculture - Effects of oxidative stress on animal welfare of juvenile turbot (*Psetta maxima*, L.). *Aquatic Toxicology,* **105,** 508-517.

Reyes-Lopez, F.E., Aerts, J., Vallejos-Vidal, E., Ampe, B., Dierckens, K., Tort, L. & Bossier, P. (2018) Modulation of Innate Immune-Related Genes and Glucocorticoid Synthesis in Gnotobiotic Full-Sibling European Sea Bass (*Dicentrarchus labrax*) Larvae Challenged With *Vibrio anguillarum*. *Frontiers in Immunology,* **9**.

Rottmann, R.W., Shireman, J.V., Starling, C.C. & Revels, W.H. (1988) Eliminating Adhesiveness of White Bass Eggs for the Hatchery Production of Hybrid Striped Bass. *The Progressive Fish-Culturist,* **50,** 55-57.

Rutala, W.A. & Weber, D.J. (2008) Guideline for Disinfection and Sterilization in Healthcare Facilities. In. Healthcare Infection Control Practices Advisory Committee (U.S.); Centers for Disease Control and Prevention (U.S.).

Salvesen, I., Skjermo, J., Vadstein, O., (2000) Microbial management in marine larviculture: perturbations in the water microfora caused by different methods of water treatment and a possible way out. In: *Aqua 2000—Responsible Aquaculture in the New Millennium 2–6 May 2000*, Nice, France.

Salvesen, I., Jørgensen, L. & Vadstein, O. (1991) Evaluation of four chemicals for surface-disinfection of marine fish eggs. In: *LARVI '91 - Fish & Crustacean larviculture symposium* (ed. by P. Lavens, P. Sorgeloos, E. Jaspers & F. Ollevier). European Aquaculture Society, special publication, Ghent, Belgium.

Salvesen, I., Oie, G. & Vadstein, O. (1997) Surface disinfection of Atlantic halibut and turbot eggs with glutaraldehyde: Evaluation of concentrations and contact times. *Aquaculture International,* **5,** 249-258.

Sanchez-Ruiz, C., Martinezroyano, S. & Tejeromonzon, I. (1995) An evaluation of the efficiency and impact of raw wastewater disinfection with peracetic acid prior to ocean discharge. *Water Science and Technology,* **32,** 159-166.

Satokari, R., Juvonen, R., Mallison, K., Von Wright, A. & Haikara, A. (1998) Detection of beer spoilage bacteria *Megasphaera* and *Pectinatus* by polymerase chain reaction and colorimetric microplate hybridization. *International Journal of Food Microbiology,* **45,** 119-127.

Schaeck, M., De Swaef, E., Van Den Broeck, W., Van Nevel, S., Boon, N., De Geyter, N., Morent, R., Demeestere, K., Duchateau, L., Coulombet, C., Haesebrouck, F. & Decostere, A. (2016a) Germ-free sea bass *Dicentrarchus labrax* larval model: a valuable tool in the study of host-microbe interactions. *Diseases of Aquatic Organisms,* **117,** 177-185.

Schaeck, M., Duchateau, L., Van Den Broeck, W., Van Trappen, S., De Vos, P., Coulombet, C., Boon, N., Haesebrouck, F. & Decostere, A. (2016b) *Vibrio lentus* protects gnotobiotic sea bass (*Dicentrarchus labrax* L.) larvae against challenge with *Vibrio harveyi*. *Veterinary Microbiology,* **185,** 41-48.

Schaeck, M., Reyes-Lopez, F.E., Vallejos-Vidal, E., Van Cleemput, J., Duchateau, L., Van Den Broeck, W., Tort, L. & Decostere, A. (2017) Cellular and transcriptomic response to treatment with the probiotic candidate *Vibrio lentus* in gnotobiotic sea bass (*Dicentrarchus labrax*) larvae. *Fish & Shellfish Immunology,* **63,** 147-156.

Schliesser, T. & Wiest, J.M. (1979) About the Temperature-Dependence of the Bactericidal Effect of Some Chemical Disinfectants. *Zentralblatt Fur Bakteriologie Mikrobiologie Und Hygiene Serie B-Umwelthygiene Krankenhaushygiene Arbeitshygiene Praventive Medizin,* **169,** 560-566.

Schrader, K.K. (2008) Compounds with inhibitory activity against the channel catrish pathogens *Edwardsiella ictaluri* and *Flavobacterium columnare*. *North American Journal of Aquaculture,* **70,** 147-153.

Schreier, T.M., Rach, J.J. & Howe, G.E. (1996) Efficacy of formalin, hydrogen peroxide, and sodium chloride on fungal-infected rainbow trout eggs. *Aquaculture,* **140,** 323-331.

Shaw, E. (1957) Potentially Simple Technique for Rearing Germ-Free Fish. *Science,* **125,** 987-988.

Sheng, Y., Ren, H., Limbu, S.M., Sun, Y.H., Qiao, F., Zhai, W.Y., Du, Z.Y. & Zhang, M.L. (2018) The Presence or Absence of Intestinal Microbiota Affects Lipid Deposition and Related Genes Expression in Zebrafish (*Danio rerio*). *Frontiers in Microbiology,* **9**.

Shepherd, J.A., Waigh, R.D. & Gilbert, P. (1988) Antibacterial Action of 2-Bromo-2-Nitropropane-1,3-Diol (Bronopol). *Antimicrobial Agents and Chemotherapy,* **32,** 1693-1698.

Shrestha, R., Joshi, D.R., Gopali, J. & Piya, S. (2009) Oligodynamic action of silver, copper and brass on enteric bacteria isolated from water of Kathmandu Valley. *Nepal Journal of Science and Technology,* **10,** 189-193.

Situmorang, M.L., De Schryver, P., Dierckens, K. & Bossier, P. (2016) Effect of poly-beta-hydroxybutyrate on growth and disease resistance of Nile tilapia *Oreochromis niloticus* juveniles. *Veterinary Microbiology,* **182,** 44-49.

Situmorang, M.L., Dierckens, K., Mlingi, F.T., Van Delsen, B. & Bossier, P. (2014) Development of a bacterial challenge test for gnotobiotic Nile tilapia *Oreochromis niloticus* larvae. *Diseases of Aquatic Organisms,* **109,** 23-33.

Small, B.C. (2004) Accounting for water temperature during hydrogen peroxide treatment of channel catfish eggs. *North American Journal of Aquaculture,* **66,** 162-164.

Small, B.C. (2009) Evaluation of Sodium Carbonate Peroxyhydrate as a Potential Catfish Egg Disinfectant. *Journal of Aquatic Animal Health,* **21,** 117-123.

Small, B.C. & Chatakondi, N. (2006) Efficacy of formalin as an egg disinfectant for improving hybrid catfish (channel catfish x blue catfish) hatching success. *North American Journal of Aquaculture,* **68,** 9-13.

Small, B.C. & Wolters, W.R. (2003) Hydrogen peroxide treatment during egg incubation improves channel catfish hatching success. *North American Journal of Aquaculture,* **65,** 314-317.

Sorensen, S.R., Skov, P.V., Lauesen, P., Tomkiewicz, J., Bossier, P. & De Schryver, P. (2014) Microbial interference and potential control in culture of European eel (*Anguilla anguilla*) embryos and larvae. *Aquaculture,* **426,** 1-8.

Stampi, S., De Luca, G. & Zanetti, F. (2001) Evaluation of the efficiency of peracetic acid in the disinfection of sewage effluents. *Journal of Applied Microbiology,* **91,** 833-838.

Stauber, J.L. & Florence, T.M. (1987) Mechanism of Toxicity of Ionic Copper and Copper-Complexes to Algae. *Marine Biology,* **94,** 511-519.

Stephenson, H., Gabel, M. & Barnes, M.E. (2005) External egg membrane characteristics of landlocked fall Chinook salmon eyed eggs treated with hydrogen peroxide or formalin. *North American Journal of Aquaculture,* **67,** 171-176.

Straus, D.L. (2008) Copper sulfate toxicity to channel catfish fry: Yolk sac versus swim-up fry. *North American Journal of Aquaculture,* **70,** 323-327.

Straus, D.L., Farmer, B.D., Ledbetter, C.K., Beck, B.H., Williams, R.S., Clark, M.L. & Freeze, T.M. (2016) Use of Copper Sulfate to Control Egg Saprolegniasis at a Commercial Sunshine Bass Hatchery. *North American Journal of Aquaculture,* **78,** 243-250.

Straus, D.L., Hossain, M.M. & Clark, T.G. (2009) Copper sulfate toxicity to two isolates of *Ichthyophthirius multifiliis* relative to alkalinity. *Diseases of Aquatic Organisms,* **83,** 31-36.

Straus, D.L., Meinelt, T., Farmer, B.D. & Mitchell, A.J. (2012) Peracetic acid is effective for controlling fungus on channel catfish eggs. *Journal of Fish Diseases,* **35,** 505-511.

Straus, D.L., Mitchell, A.J., Carter, R.R. & Steeby, J.A. (2009) Optimizing Copper Sulfate Treatments for Fungus Control on Channel Catfish Eggs. *Journal of Aquatic Animal Health,* **21,** 91-97.

Straus, D.L., Mitchell, A.J., Carter, R.R. & Steeby, J.A. (2012) Hatch rate of channel catfish *Ictalurus punctatus* (Rafinesque 1818) eggs treated with 100 mg L-1 copper sulphate pentahydrate. *Aquaculture Research,* **43,** 14-18.

Straus, D.L., Mitchell, A.J., Radomski, A.A., Carter, R.R. & Steeby, J.A. (2009) Laboratory Dose Confirmation of Copper Sulfate for Treating Fungus on Channel Catfish Eggs. *North American Journal of Aquaculture,* **71,** 333-338.

Stuart, K.R., Keller, M. & Drawbridge, M. (2010) Efficacy of formalin and povidone-iodine disinfection techniques on the eggs of three marine finfish species. *Aquaculture Research,* **41,** e838-e843.

Stuart, N.C. (1983) Treatment of Fish Disease. *Veterinary Record,* **112,** 173-177.

Subasinghe, R. & Sommerville, C. (1985) Disinfection of *Oreochromis mossambicus* (Peters) eggs against commonly occurring potentially pathogenic bacteria and fungi under artificial hatchery conditions. *Aquaculture Research,* **16,** 121-127.

Sun, Q., Hu, K. & Yang, X.L. (2014) The Efficacy of Copper Sulfate in Controlling Infection of *Saprolegnia parasitica*. *Journal of the World Aquaculture Society,* **45,** 220-225.

Takigawa, T. & Endo, Y. (2006) Effects of glutaraldehyde exposure on human health. *Journal of Occupational Health,* **48,** 75-87.

Tendencia, E.A. (2001) Effect of iodine disinfection on the bacterial flora and hatching rate of grouper, *Epinephelus coioides* eggs at the cleavage and eyed stages. *Bulletin of the European Association of Fish Pathologists,* **21,** 160-163.

Treasurer, J.W., Cochrane, E. & Grant, A. (2005) Surface disinfection of cod *Gadus morhua* and haddock *Melanogrammus aeglefinus* eggs with bronopol. *Aquaculture,* **250,** 27-35.

Verner-Jeffreys, D.W., Nakamura, I. & Shields, R.J. (2007) Surface disinfection of Pacific threadfin, *Polydactylus sexfilis*, and amberjack, *Seriola rivoliana*, eggs. *Aquaculture Research,* **38,** 605-612.

Verner-Jeffreys, D.W., Shields, R.J. & Birkbeck, T.H. (2003) Bacterial influences on Atlantic halibut *Hippoglossus hippoglossus* yolk-sac larval survival and start-feed response. *Diseases of Aquatic Organisms,* **56,** 105-113.

Wagner, E.J., Bartley, M.S. & Oplinger, R.W. (2012) Egg Disinfection to Improve Conservation Aquaculture of Leatherside Chub. *North American Journal of Aquaculture,* **74,** 199-207.

Wagner, E.J. & Oplinger, R.W. (2013) Toxicity of Copper Sulfate to *Flavobacterium psychrophilum* and Rainbow Trout Eggs. *Journal of Aquatic Animal Health,* **25,** 125-130.

Wagner, E.J., Oplinger, R.W., Arndt, R.E., Forest, A.M. & Bartley, M. (2010) The Safety and Effectiveness of Various Hydrogen Peroxide and Iodine Treatment Regimens for Rainbow Trout Egg Disinfection. *North American Journal of Aquaculture,* **72,** 34-42.

Wagner, E.J., Oplinger, R.W. & Bartley, M. (2012) Effect of Single or Double Exposures to Hydrogen Peroxide or Iodine on Salmonid Egg Survival and Bacterial Growth. *North American Journal of Aquaculture,* **74,** 84-91.

Wagner, E.J., Oplinger, R.W. & Bartley, M. (2012) Evaluation of Tannic Acid for Disinfection of Rainbow Trout Eggs. *North American Journal of Aquaculture,* **74,** 80-83.

Wagner, E., Oplinger, R. & Bartley, M. (2012d) Penicillin-G: Efficacy against *Flavobacterium psychrophilum* and evaluation of lethal dose limits for rainbow trout. *Open Journal of Animal Sciences*, **2**, 150-158.

Walker, A.B., Ward, D., Duclos, K., Peters, M. & Berlinsky, D.L. (2010) Surface Disinfection and Removal of Adhesiveness from Rainbow Smelt Eggs. *North American Journal of Aquaculture,* **72,** 158-163.

Watanabe, M. (1942) Salmon Culture in Japan - The Chitose Salmon Hatchery. *Progressive Fish-Culturist,* **48,** 14-18.

Waterstrat, P.R. & Marking, L.L. (1995) Clinical-Evaluation of Formalin, Hydrogen Peroxide, and Sodium Chloride for the Treatment of *Saprolegnia Parasitica* on Fall Chinook Salmon Eggs. *Progressive Fish-Culturist,* **57,** 287-291.

Wedemeyer, G.A., Nelson, N.C. & Smith, C.A. (1978) Survival of Salmonid Viruses Infectious Hematopoietic Necrosis (Ihnv) and Infectious Pancreatic Necrosis (Ipnv) in Ozonated, Chlorinated, and Untreated Waters. *Journal of the Fisheries Research Board of Canada,* **35,** 875-879.

White, T. (1957) Tannins—their occurrence and significance. *Journal of the Science of Food and Agriculture,* **8,** 377-385.

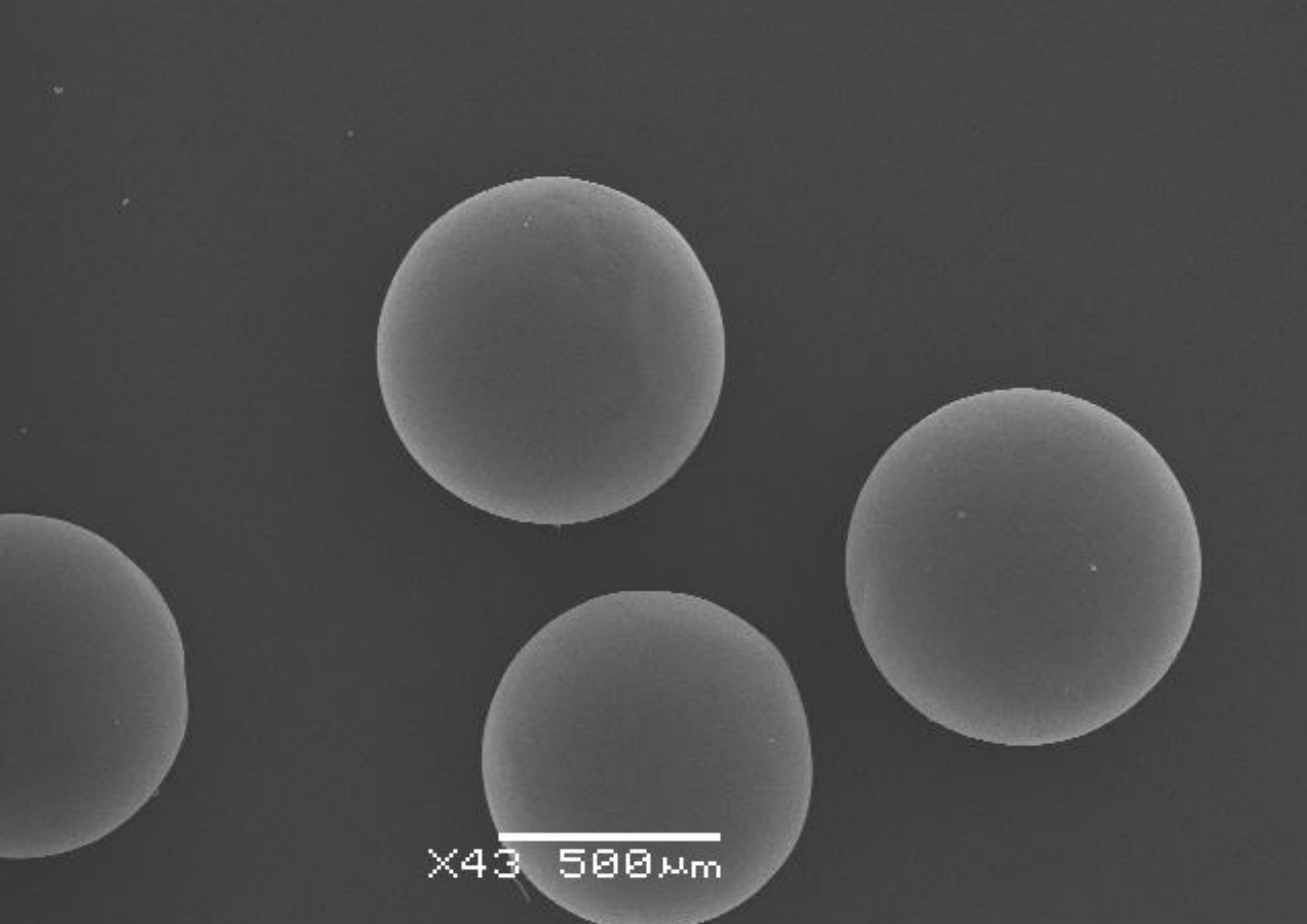
Wilson, I.G. (1997) Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology,* **63,** 3741-3751.

Wright, L.D. & Snow, J. (1975) The effect of six chemicals for disinfection of largemouth bass eggs. *The Progressive Fish-Culturist,* **37,** 213-217.

Yaacob, E.N., De Geest, B.G., Goethals, J., Bajek, A., Dierckens, K., Bossier, P. & Vanrompay, D. (2018) Recombinant ferritin-H induces immunosuppression in European sea bass larvae (*Dicentrarchus labrax*) rather than immunostimulation and protection against a *Vibrio anguillarum* infection. *Veterinary Immunology and Immunopathology,* **204,** 19-27.

Yaacob, E.N., Goethals, J., Bajek, A., Dierckens, K., Bossier, P., De Geest, B.G. & Vanrompay, D. (2017) Preparation and Characterization of Alginate Microparticles Containing a Model Protein for Oral Administration in Gnotobiotic European Sea Bass (*Dicentrarchus labrax*) Larvae. *Marine Biotechnology,* **19,** 391-400.

Zhao, G.J., Chung, K.T., Milow, K., Wang, W.X. & Stevens, S.E. (1997) Antibacterial properties of tannic acid and related compounds against the fish pathogen *Cytophaga columnaris*. *Journal of Aquatic Animal Health,* **9,** 309-313.


X43 500µm

Chapter 4 ULTRASTRUCTURAL MORPHOLOGY OF THE ENVELOPE OF DOVER SOLE *SOLEA SOLEA* EGGS FROM FERTILIZATION UNTIL HATCHING WITH EMPHASIS ON SAMPLE PREPARATION

Based on: De Swaef, E., Claeys, M., Bert, W., Huysseune, A., Witten, P.E., Van den Broeck, W. & Decostere, A. (2017) Ultrastructural morphology of the envelope of Dover sole *Solea solea* eggs from fertilization until hatching with emphasis on sample preparation. *Micron*. **99**, 9-18.

4.1 ABSTRACT

This study is the first to describe the ultrastructural morphology of the envelope of Dover sole *(Solea solea)* eggs from fertilisation until hatching. Defining the ultrastructural morphology of fish eggs is important for species identification and may assist in predicting the effect of external influences on these early life stages. In first instance, various fixation and embedding protocols were assessed to explore the morphology of the egg envelope, whereby the encountered difficulties were highlighted. The successful protocol for SEM proved to be combined fixation with 4% glutaraldehyde in 0.1 M cacodylate buffer for minimum 4 h with post-fixation of 2 h with 1% OsO_4 in 0.1 M cacodylate buffer. For TEM, puncturing the egg envelope during the first steps of the fixation protocol was necessary to allow the embedding medium to penetrate through the egg envelope. Based on both scanning and transmission electron microscopical examination, three distinct layers were discerned in the egg envelope. During the development of the fish embryo, a change in the outer structure of the egg was observed. Scanning electron microscopical examination of one day post-fertilisation eggs (DPF) revealed a homogeneous outer layer, displaying a large number of pores uniformly distributed on the surface of the egg envelope. Starting from 2 DPF parts of the outermost layer or two outer layers peeled off. The second deeper layer showed larger pores, with less defined edges. In the third innermost layer irregular indentations were noted. On transmission electron microscopy the first outermost layer of 1 DPF eggs clearly folded into the pores. The second layer was more electron dense, had a uniform appearance and did not cover the surface of the pores. The third innermost layer was much thicker and possessed indentations. A total number of 12 undulating zones were discriminated based on different degrees of electron density. Prior to hatching, the compact structure of the innermost layer was distorted by dispersed holes and tears.

4.2 INTRODUCTION

To meet the rising demand for fish induced by the continuously growing human population, aquaculture has expanded very rapidly and is now the fastest growing food producing industry in the world. Fish and fishery products represent a widespread, affordable and healthy source of protein. With stagnating global capture fishery production, aquaculture has the potential to produce higher amounts of safe and quality fish in the future, to meet the increasing demand for (sea)food (FAO, 2014). The growing importance of the aquaculture sector during recent decades combined with indications of market saturation for some established species like sea bass (*Dicentrarchus labrax*), render a diversification of fish species and products imperative (FAO, 2014). Flatfish species constitute important and credible new aquaculture candidates as exemplified by Dover sole (*Solea solea*) exhibiting high flesh quality and important market value, and being the subject of an increased consumer demand (Imsland et al., 2003). As for other cultured marine teleost species, production has been severely hampered by difficulties in larval rearing as manifested by low and unpredictable survival rates of both eggs and larvae (Boglione et al., 2013a; 2013b). Dover sole is considered to have a moderate vulnerability to (local) extinction caused by fisheries (Cheung et al., 2005) and produces pelagic eggs, which hatch around 1400 degree hours (at 13 °C) after fertilisation (Devauchelle et al., 1987). Despite its growing significance, no information is available on the egg structure and composition, which could assist in further understanding the development of Dover sole and improve its chances for obtaining the status of a successful aquaculture species.

Overfishing and destruction of marine habitats especially affects benthic fish populations as indicated by landings for plaice (*Pleuronectes platessa*) that declined more than 97% (Thurstan et al., 2010). Today, electrofishing is a promising alternative fishing technique for beam trawling when targeting flatfish and shrimp to mitigate at least the destruction of benthic habitats. The possible effects on marine organisms have been extensively studied during recent years (Soetaert et al., 2013) with the exception of the impact of exposing early developmental stages, including eggs, to electrical pulses. Such data are crucial, as electrofishing may occur over these shallow active spawning areas of many species, including Dover sole. Earlier studies on fresh water species have indicated a reduced survival of embryos if exposure to the electric field happened during the first embryonic stages, in particular around early cleavage and epiboly (Godfrey 1957; Dwyer et al., 1993; Muth & Ruppert, 1997; Henry & Grizzle, 2004; Bohl et al., 2010). Bohl et al. (2010) suggested that the effect on survival was not related to the species differences in themselves, but to physical characteristics (i.e. size) of the eggs rendering a prediction of the vulnerability in untested species possible based upon morphological data.

The teleost egg is surrounded by a complex multi-layered envelope, composed of protein fibres in a protein matrix (Iconomidou et al., 2000) with a specific (ultra)structure and chemical composition. In

addition, the structure and thickness of the egg envelope also varies depending on the developmental stage of the egg or the ecological conditions of the parental population (Chen et al., 1999). The egg envelope undergoes structural and biochemical changes during oocyte maturation (Ravaglia & Maggese, 2003; Fausto et al., 2004; Bian et al., 2010). Furthermore, immediately after fertilisation large changes in egg morphology occur to prevent polyspermy and to protect the developing embryo (Iwamatsu & Ohta, 1981; Kobayashi & Yamamoto, 1981; Iwamatsu et al., 1991; 1993; Mekkawy & Osman, 2006). The most important proteins and glycoproteins of the egg envelope have been characterized in different species (Scapigliati et al., 1994, 1995; Oppen-Bernsten et al., 1990; Baldacci et al., 2001). More recently, changes in these proteins are linked to environmental pollutants, making the egg envelope a potential sensitive biomarker (Arukwe et al., 1997; Arukwe & Goksöyr, 2003; Berois et al., 2011). In addition, the ultrastructural morphology of both the outer surface of the egg envelope and of the micropyle are frequently used for species identification and phylogenetic classification (Riehl, 1980; Lønning et al., 1988; Chen et al., 1999; Li et al., 2000; Gwo, 2008), again reinforcing the importance of the availability of these morphological data.

Considering the above, the aim of this study is to investigate the overall ultrastructural morphology of the egg envelope of Dover sole embryos during development.

There is considerable variation in the nomenclature used to describe the external layers of teleost eggs, hampering comparison between studies. Frequently used terms are zona radiata (Breining et al., 2000), chorion (Lønning & Davenport, 1980; Chen et al., 2007), egg membrane (Stehr & Hawkes, 1979), vitelline envelope (Ravaglia & Maggese, 2003), egg envelope (Scapigliati et al., 1994) and egg shell (Baldacci et al., 2001). In this study, the term egg envelope was used when referring to the outermost layers of the fertilised fish egg, surrounding the fish embryo. To describe the openings in the outer layers of the egg envelope, the term pore was employed which coincides with most of the literature relevant in this domain.

4.3 MATERIAL AND METHODS

4.3.1 Sample collection

Solea solea embryos were obtained from the Institute for Marine Resources & Ecosystem Studies (IMARES) in IJmuiden, the Netherlands. Eggs were naturally spawned, fertilised overnight and collected the next morning. Following transportation in natural seawater with a salinity of 32 ppt, embryos were acclimatized with and further incubated in aerated artificial seawater (Instant Ocean Aquarium Systems, Mentor, Ohio; salinity 34 ppt; 9 h light/15 h darkness). Embryos were determined as developing when the eggs remained floating (dead eggs sank to the bottom of the tank when aeration was discontinued) and no white discoloration was observed. Only developing embryos were sampled.

For scanning electron microscopy, embryos were incubated at 17 ± 1 °C and sampled 1 day post-fertilisation (DPF), 2 DPF and 3 DPF. Hatching followed during the night between 3 DPF and 4 DPF. For transmission electron microscopical examination, embryos were incubated at 15 ± 1 °C and sampled on 1 DPF, 2 DPF, 3 DPF and 4 DPF. Hatching occurred during the following night.

4.3.2 Scanning electron microscopy

Various fixation protocols in terms of fixative constituents (single and in combination) and concentration and fixation times were tested on minimal 20 embryos 1 DPF (Table 4.1, Table 4.2). After fixation, the embryos were washed three times with cacodylate buffer (0.1 M). Post-fixation with 2% osmium tetroxide (Os_2O_4) followed by washing three times with cacodylate buffer (0.1 M) was an optional further step. Thereafter, dehydration of the embryos was performed in a graded series of ethanol (10 to 95% in water, Table 4.2), followed by a graded series of acetone (10 to 100% in ethanol, Table 4.2). Finally, samples were placed in 100% acetone and incubated at room temperature overnight. The next day, the embryos were dried with a Balzers CPD 030 critical-point dryer (Leica, Diegem, Belgium) and platinum-coated using a JEOL JFC-1300 Auto Fine Coater (Jeol Ltd, Zaventem, Belgium). The specimens were observed and photographed with a JEOL JSM 5600 LV scanning electron microscope (Jeol Ltd) under an accelerating voltage of 15kV.

Stereomicroscopic examination of the embryos was performed after fixation and both following dehydration with ethanol and acetone. Only those protocols resulting in at least 75% of the embryos being fully round were further employed (Table 4.1). The protocols that, having gone through all steps, yielded round, intact embryos for SEM were repeated twice in time, using different embryo batches of the same age.

To determine the size of the embryos, 24 fertilized embryos of each sampling day were examined based on the SEM images. For each embryo, the best fitting circle was drawn and its diameter measured with Scandium 5.2 software (Soft Imaging System, Olympus N.V.). This method was also used to establish the micropyle diameter. The difference in embryo diameter between the three sampling days was tested using one-way ANOVA, post hoc comparisons were performed using a Tukey HSD test. Statistical results were considered significant when p-values < 0.01. The density of pores was determined for 26 intact embryos of 1 DPF, based on SEM images. A detailed image of the surface of the egg envelope of these embryos was made, a 10 x 10 µm grid placed randomly in the central region of the egg envelope and the pores counted.

4.3.3 Transmission electron microscopy

Three different embedding protocols for TEM examination of the fertilized eggs were tested (Table 4.3), all initiating with fixation in 4% glutaraldehyde in 0.1 M cacodylate buffer. In all protocols the embedding medium (SPURR or Epon) was added in low amounts to allow slow penetration and avoid

embryos collapsing. To enhance penetration, embryos in protocols 2 and 3 were punctured once with a glass capillary under a stereomicroscope. Puncturing was conducted at the start (protocol 3) or after (protocol 2) fixation depending on the protocol (Table 4.3). Semi (0.5 µm)- and ultrathin (80 nm) sections were produced using a Reichert Jung Ultracut E ultramicrotome (Reichert Jung Inc., Buffalo, NY, USA) with a diamond knife (Diatome Ltd., Biel, Switzerland) and collected on copper single-slot grids (Agar Scientific, Stansed, UK) coated with formvar film. Post-staining of the sections was carried out for 30 min in uranyl acetate and for 7 min in lead citrate using a Leica EM AC20 automatic contrasting instrument (Leica Microsystems, Wetzlar, Germany). Sections were studied with a Jeol JEM-1010 transmission electron microscope (Jeol Ltd., Tokyo, Japan) operating at 60kV, and images were digitized using a Ditabis system (Pforzheim, Germany). For the final photographs the JEM-1400 plus Jeol electron microscope (Jeol Ltd.) operating at 80kV was used and micrographs were taken digitally. The embedding protocol yielding round, intact embryos with an adequately fixed egg envelope and well preserved yolk, as indicated by complete ultrathin sections, was selected for processing the fertilised eggs of different developmental stages for TEM examination. Measurements of egg envelope thickness were performed on three embryos per sampling day using Cell D software (Soft Imaging System, Olympus NV). The difference in egg envelope thickness between the three sampling days was tested using one-way ANOVA, statistical results were considered significant when p-values < 0.01.

4.4 RESULTS

4.4.1 Scanning electron microscopy

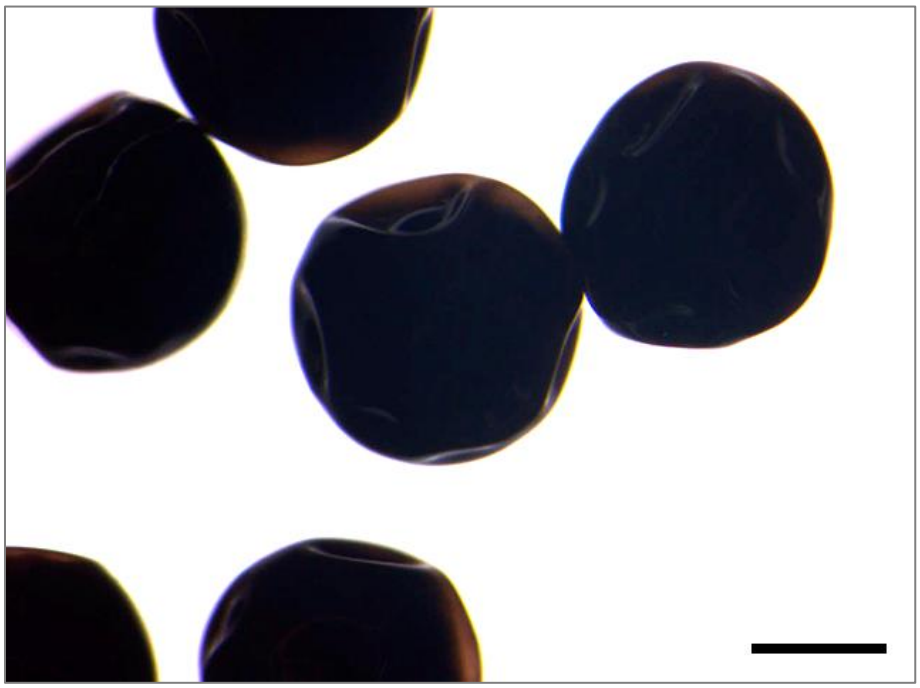


Figure 4.1 - Stereomicroscopic view of collapse of the Dover sole embryos after dehydration with ethanol (protocol 4a). Bar = 500 µm.

Although all fixatives initially resulted in round fertilised eggs, following dehydration with ethanol and aceton many caused embryo collapse and shrinkage (Figure 4.1), leading to their elimination for further testing (Table 4.1). Of the remaining seven protocols, only three protocols (9a, 9b and 18c) resulted in well fixated, round embryos after completion of all steps (Figure 4.2). These protocols were repeated twice in time, all three giving reproducible results. As protocol 9b did not include post-fixation with OsO_4, necessary for TEM, and protocol 18 involved more fixative constituents, protocol 9a was chosen for further use. The latter protocol combined fixation with 4% glutaraldehyde in 0.1 M cacodylate buffer for minimum 4 h with post-fixation of 2 h with 1% OsO_4 in 0.1 M cacodylate buffer.

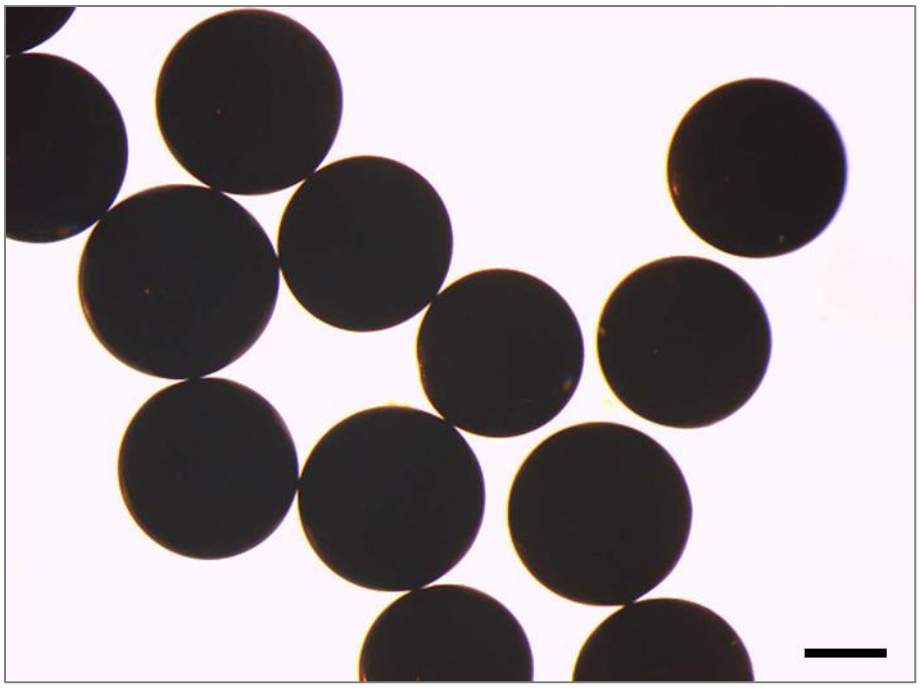


Figure 4.2 - Stereomicroscopic view of round fixated Dover sole embryos (protocol 9a). Bar = 500 µm.

The average embryo diameter was 843.08 ± 23.76 µm, 803.54 ± 51.92 µm and 795.54 ± 33.32 µm, 1 DPF, 2 DPF and 3 DPF, respectively. A significant decrease in diameter could be observed between 1 DPF and 2 DPF ($p < 0.01$), and between 1 DPF and 3 DPF ($p < 0.01$).

Scanning electron microscopy of 1DPF embryos showed one homogeneous outer layer, containing a large number of pores uniformly distributed over the surface of the egg envelope (Figure 4.3 and 4.4). Pore distribution density was 22.0 (± 1.5) /100 µm^2. In one of the fertilized eggs a funnel shaped micropyle with a diameter of 11.61 µm was visible. Pore distribution remained similar in this area. Scanning electron microscopy of 2 DPF embryos revealed an irregular appearance. At low magnification, separation of the egg envelope layers was visible. Depending on the region, parts of the first outermost layer or the two outer layers peeled off, resulting in a flaked appearance of the surface. At higher magnification, parts of the first layer could be seen with evenly distributed pores. The deeper second layer showed larger pores, with less defined edges. In the innermost third layer, no actual pores were observed but irregular indentations were noted across the surface of this layer (Figure 4.3). Again, in one of the fertilized eggs a funnel shaped micropyle was found with a diameter of 9.13 µm (Figure 4.4).

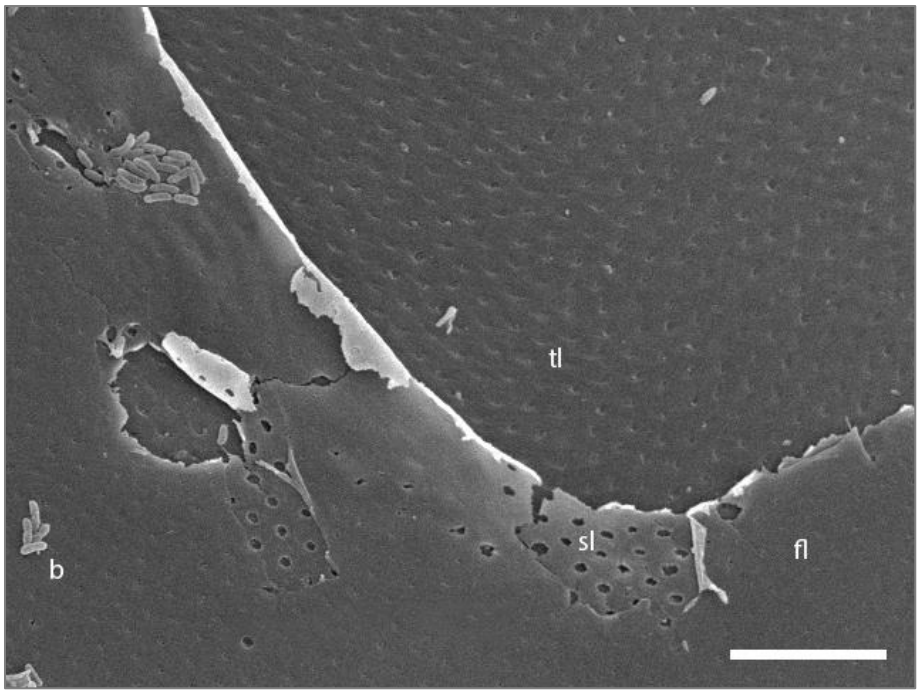


Figure 4.3 - Peeling off of different envelope layers in Dover sole embryos sampled at 2 DPF (SEM). fl: first outermost layer of the egg envelope, sl: second deeper layer of the egg envelope, tl: third innermost layer of the egg envelope, b: bacterial cells. Bar = 10 µm.

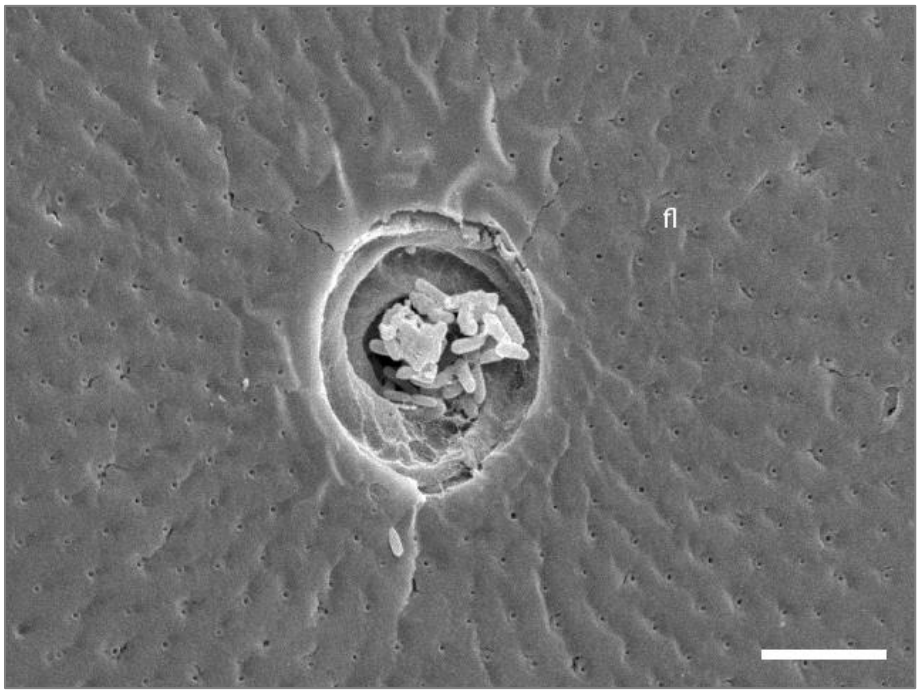


Figure 4.4 - Funnel shaped micropyle of Dover sole embryos sampled at 1 DPF (SEM). Rod shaped bacterial cells are visible. fl: first outermost layer of the egg envelope. Bar = 5 µm.

Scanning electron microscopy of 3 DPF embryos revealed that the outer layers were peeled off even more. The first, outermost layer was only observed in very small areas on the egg envelope surface.

The latter mostly consisted of the innermost (third) layer, with irregular indentations but small regions with fragments of the second layer were locally present. In other regions of the egg envelope, no even layer was found, with only remnants of egg shell material noticeable. In one of the 3 DPF embryos the funnel shaped micropyle of one embryo was also visible. The micropylar opening of the channel was 9.14 µm in diameter. The area surrounding the micropyle showed a different distribution pattern of pores. Pores enlarged and were directed towards the micropyle (Figure 4.5).

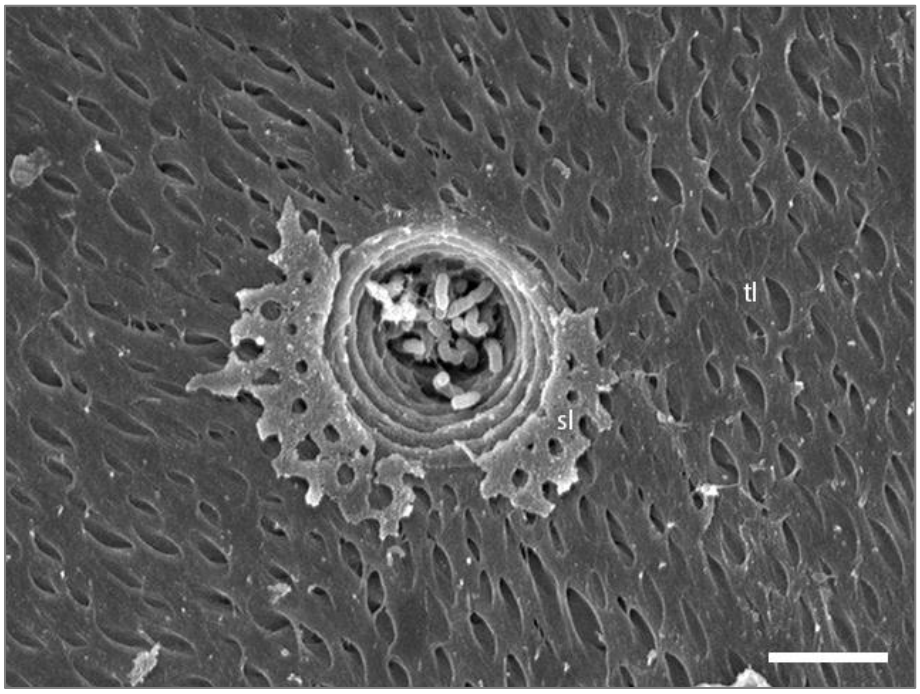


Figure 4.5 - Funnel shaped micropyle of Dover sole embryos sampled at 3 DPF (SEM). Rod shaped bacterial cells are visible. sl: second deeper layer of the egg envelope, tl: third innermost layer of the egg envelope. Bar = 5 µm.

Small groups of aggregated short rod shaped bacterial cells were distinguished on all sampled embryos and within the observed micropyles. No apparent increase in the amount of bacterial cells during development was noted.

4.4.2 Transmission electron microscopy

For TEM, protocol 1 (Table 4.3) caused complete collapse of the fertilized eggs when the material came in contact with the embedding medium (SPURR). Although protocol 2 (Table 4.3) resulted in a much better morphology and round embryos were retrieved, poor preservation of the yolk prevented obtaining intact ultrathin sections. Protocol 3 (Table 4.3) resulted in round, intact egg envelope with well-preserved yolk, necessary to produce good ultrathin sections.

The layered structure of the egg envelope noticed by SEM was also observed upon TEM examination, although no peeling off of the different layers could be observed. In the ultrathin section of 1 DPF

embryos blindly ending pores were noticeable, consistent with the SEM observations (Figure 4.6). Pores were 'plugged' with amorphous semi-translucent material and three layers of the egg envelope were distinguished (Figure 4.6 & 4.7). The first outermost layer was clearly folding into the pores. The second layer had a uniform appearance not covering the surface of the pores. The darker colour of this layer indicated a more electron dense structure. The third innermost layer was thicker (Table 4.4) and exhibited indentations. Different electron dense and electron lucent zones were discriminated. A total number of 12 undulating zones were encountered (Figure 4.6). Underneath these layers the perivitelline space was noticeable, marking the boundary of the egg envelope.

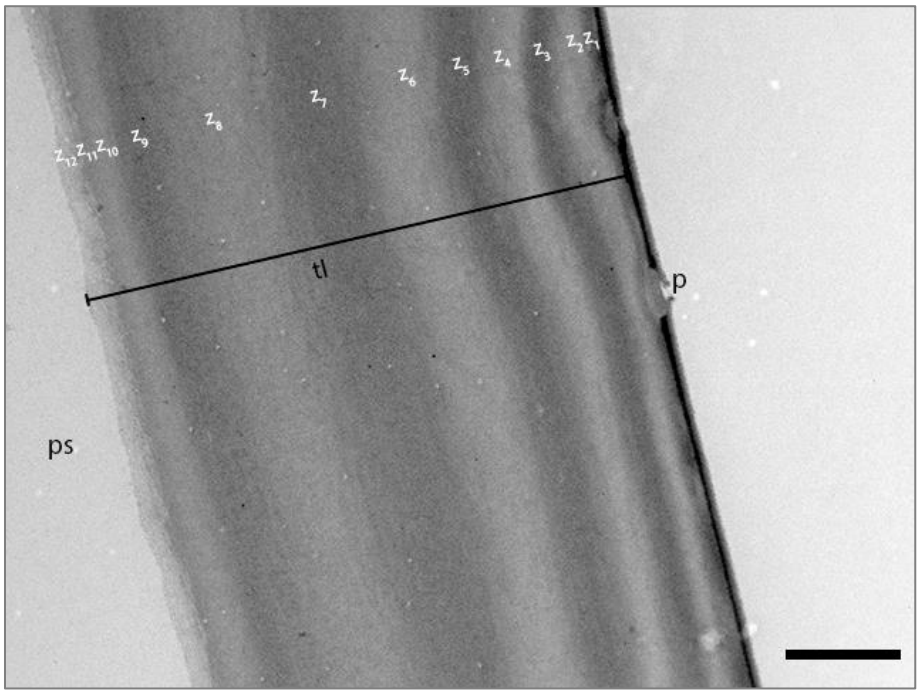


Figure 4.6 - Ultrathin sections of the egg envelope of Dover sole embryos (1 DPF), showing three distinct layers. (TEM). tl: third innermost layer of the egg envelope consisting of 12 zones of alternating electrondensity (Z1 till Z12), ps: perivitelline space, p: pore. Bar = 2 µm.

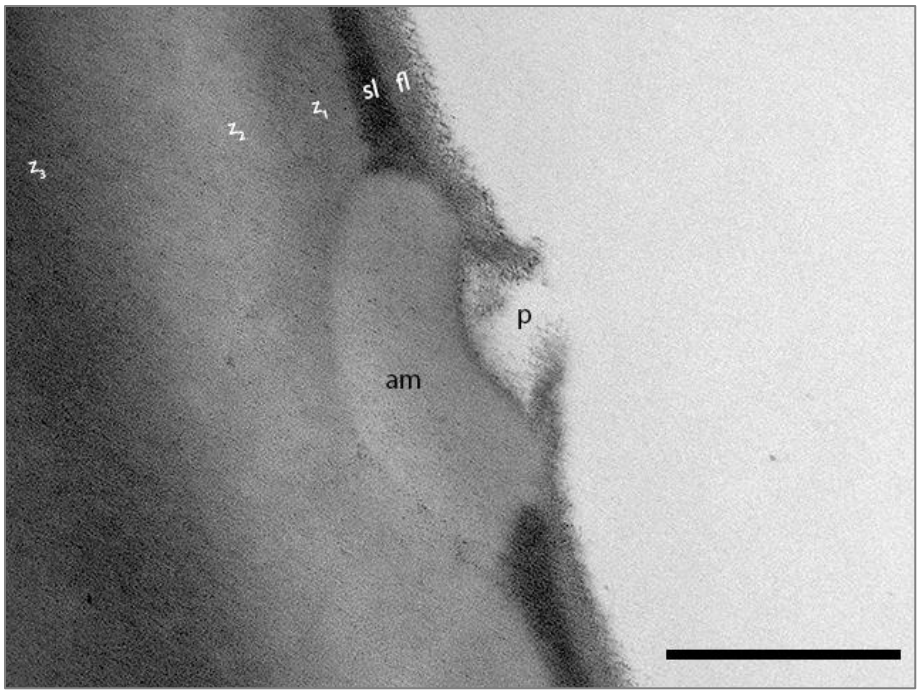


Figure 4.7 - Detail of a pore in the external layers of the egg envelope of the Dover sole embryo (1 DPF), showing a plug of amorphous semi-translucent material (TEM). fl: first outermost layer of the egg envelope, sl: second deeper layer of the egg envelope, z1-z3: zones of alternating electrondensity of the third innermost layer, am: amorphous material, p: pore. Bar = 500 nm.

Ultrathin sections of 2 DPF and 3 DPF embryos provided the same appearance (Figure 4.8). For both days three layers as noted for the first day, were observed. The only discernible difference was the disappearance of 'plug' material inside the pores.

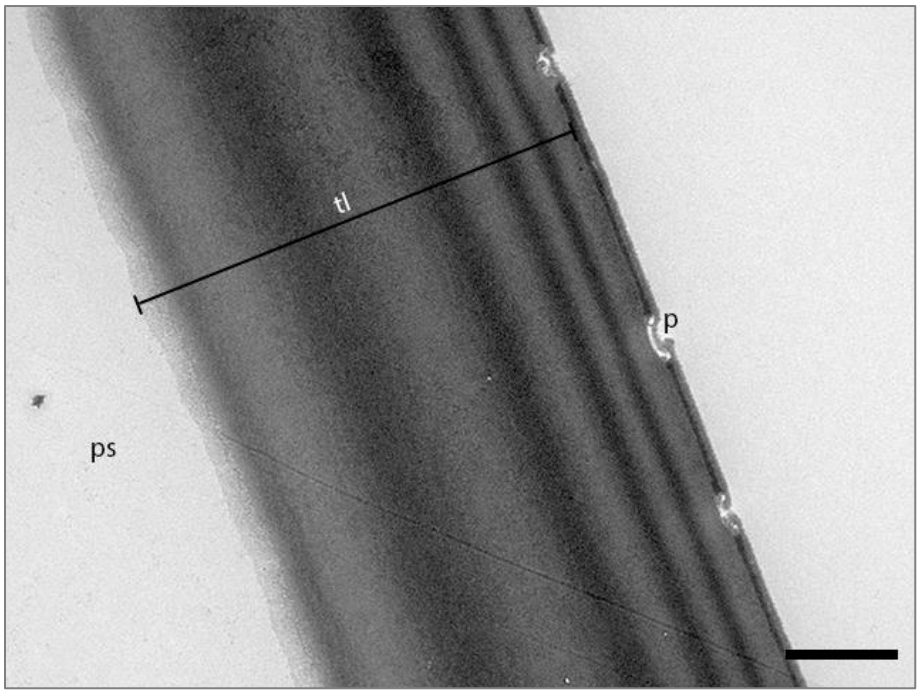


Figure 4.8 - Detail of the external layers of the egg envelope of the Dover sole embryo (3DPF), showing three distinct layers (TEM). No plug of amorphous material is present in the pores. tl: third innermost layer of the egg envelope, p:pore, ps: perivitelline space, p: pore. Bar = 2 µm.

Transmission electron microscopical examination of 4 DPF embryos revealed the same layered structure, but there was a clear change in the overall compact and continuous structure of the egg envelope (Figure 4.9). Large openings underneath the pores were visible. In addition, many holes and tears were scattered in different areas throughout in the underlying deeper zones. No significant differences in thickness of the three egg envelope layers during embryonic development could be observed (Table 4.4).

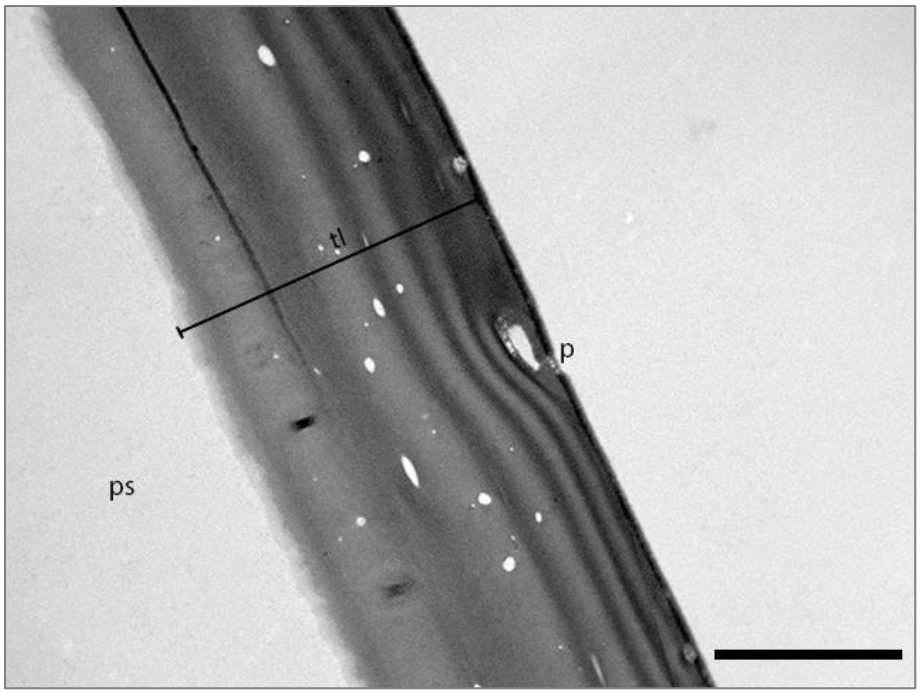


Figure 4.9 - Detail of the external layers of the egg envelope of the Dover sole embryo (4DPF), showing three distinct layers (TEM). Holes are visible in the third innermost layer. tl: third innermost layer of the egg envelope, ps: perivitelline space, p: pore. Bar = 5 µm.

4.5 DISCUSSION

As a prerequisite for the ultrastructural examination of Dover sole embryos, the fixation protocol needed to be pinpointed. For TEM, puncturing the egg envelope during the first steps of the fixation protocol was necessary to allow the embedding medium to penetrate through the egg envelope (Table 4.3), as was noted for nematode eggs (Vancoppenolle et al., 2000) and in two studies involving fish eggs (Stehr & Hawkes, 1979; Lønning & Davenport, 1980). These extra steps enabling TEM examination were not necessary in the vast majority of studies involving other fish species (e.g. Fausto et al., 1994; Morrison et al., 1999; Faustino et al., 2010), underscoring the interspecies differences.

The uniformly distributed pores observed in the outermost layer of the embryo 1 DPF were also observed for other marine species. Especially, but not exclusively, flatfish appear to have a similar morphology in terms of pore distribution under SEM (Hagström & Lønning, 1968; Stehr & Hawkes, 1979; Lønning & Davenport, 1980; Hirai, 1988; Bian et al., 2010), with no ornamentation of the egg envelope, as seen in other roundfish species (Riehl & Kokoscha, 1993; Breining & Britz, 2000; Fava & Toledo-Piza, 2007). This ornamentation has been considered to be important for demersal eggs, enabling adhesion to submerged plants and facilitating attachment to structures on the sea floor (Rizzo et al., 2002). Demersal eggs also need a better protection against mechanical stress, which raises the need for a more complex, thick egg envelope (Stehr & Hawkes, 1979; Lønning et al., 1988). In contrast,

pelagic eggs tend to have thin egg envelopes and a simple composition, as this would improve buoyancy (Stehr & Hawkes, 1979; Rizzo et al., 2002). This is consistent with the results for Dover sole.

Since the absolute size of the fertilized eggs may be affected during SEM preparation, the measurements should be regarded as an approximate value. However, a significant difference in size between embryos of 1 DPF and 3 DPF was noted. Furthermore, the peeling off of the outer layers (Figure 4.3 and 4.5) may have influenced size estimates. More pronounced, swelling followed by a decrease in diameter was also observed in *H. platessoides limandoides* (Lønning & Davenport, 1980), pointing towards a change in the morphology during embryonic development which is often referred to as egg activation (Lønning & Davenport, 1980) or water hardening (Jeon & Friedlander, 1992). The ability to swell might also explain the undulating character of the various zones of differing electrondensity (Figure 4.7-4.9) observed in the innermost layer, a feature already noted in other studies, although the zone number (sometimes called lamellae) varied greatly between species (Lønning, 1972).

Peeling off of the layers was described for various marine fish species and attributed to the exoenzymatic activity of the bacteria associated with the egg surface (Lønning & Davenport, 1980; Hansen & Olafsen, 1989; Morrison et al., 1999). For Dover sole embryos, a smaller number of aggregated short rod shaped bacterial cells was noted on the egg envelope (Figure 4.3-4.5) compared to the former studies. Dover sole embryos were incubated in artificial seawater after arrival and dead eggs/embryos were removed daily. Therefore, bacterial densities were probably much lower compared with natural seawater conditions, explaining the limited number of bacterial cells on the SEM images. Furthermore, all embryos underwent a long series of manipulations, which may have flushed away the largest proportion of loosely adherent bacteria. An alternative hypothesis for the peeling off of the layers, is that an alternation in the structure of the envelope related to the prospective hatching takes place. The peeling off of the layers was only visible for SEM and not TEM, which may be explained by the additional drying step (including high pressure and low temperatures) in the former. Nevertheless, all embryos for SEM were analysed in the same manner, indicating an actual change in the egg envelope structure during embryonic development.

In previous morphological studies authors used mostly unfertilised eggs sampled once (Fausto et al., 1994; Li et al., 2000; Baldacci et al., 2001; Gwo, 2008), rendering our study where various developmental stages were sampled, unique. When the actual morphological changes associated with fertilisation were studied, sampling took place at several time points (within minutes or hours) around this event (Iwamatsu et al., 1991; Mekkawy & Osman, 2006). To our knowledge, frequent sampling between fertilisation and hatching was only done for *H. platessoides limandoides* but the report does not include a fully detailed description of SEM and TEM (Lønning & Davenport, 1980). Studying the egg

envelope during the development of the embryo underlines the added value of this study as hereby, intriguing differences in between the embryonic stages were revealed that may impact diagnostics. Moreover, upon identifying wild eggs, the majority will be fertilised hampering the use of the published diagnostic characteristics of unfertilised eggs (Fausto et al., 1994; Chen et al., 1999; Li et al., 2000; Baldacci et al., 2001). Additionally, the widely used characteristics of micropylar canals are unsuitable for diagnostic analyses of fertilised eggs, as they change dramatically after fertilisation (Iwamatsu et al., 1991; Chen et al., 2007).

4.6 ACKNOWLEDGEMENTS

Prof. dr. Paul Simoens is gratefully acknowledged for critically reviewing the manuscript. Furthermore, the authors would like to thank Bart De Pauw and Mieke Soenens for technical support and IMARES Wageningen UR for providing the *S. solea* eggs. This work was supported by the Special Research Grant [Bijzonder Onderzoeksfonds, grant number BOF12/GOA/022, BOF12/BAS/070] of Ghent University, Belgium and the Hercules foundation [grant number AUGE/11/009].

Table 4.1 - Overview of the tested fixation protocols, the constituents, fixation time and the optional step of post-fixation with osmium tetroxide (OsO_4). Subsequent protocol steps (1, 2, 3) were continued in the stages indicated with 'X', and halted when less than 75% fully round eggs were obtained.

Protocol number	Constituents	Fixation time	Postfixation with OsO_4	Completed protocol steps			Reference
				1. Series of ethanol	2. Series of aceton	3. Critical point drying and sputtering	
1a	0.1 M phosphate buffer saline (PBS) (pH 7.4) + 2.5% glutaraldehyde	36 h	2 h	X			1, 2
1b	PBS + 2.5% glutaraldehyde	36 h	/	X	X		1*
2a	PBS + 3% glutaraldehyde	36 h	2 h	X			1*, 3*
2b	PBS + 3% glutaraldehyde	36 h	/	X	X		1*
3a	PBS + 1% glutaraldehyde+ 4% formaldehyde	36 h	2 h	X			1*
3b	PBS + 1% glutaraldehyde+ 4% formaldehyde	36 h	/	X			1*
4a	PBS + 3% glutaraldehyde + 2% Alcian blue (pH 2.5)	15 h	2 h (in PBS)	X			1*, 3*
4b	PBS + 3% glutaraldehyde + 2% Alcian blue (pH 2.5)	15 h	/	X	X	X	1*
5a	4% glutaraldehyde in 0.2 M cacodylate buffer	36 h	2 h	X	X		4*
5b	4% glutaraldehyde in 0.2 M cacodylate buffer	36 h	/	X			4*
6	OsO_4 (2%) dissolved in seawater	2 h	/	X			4*, 5*
7a	Hepes fixative ½ diluted	36 h	2 h	X	X		
7b	Hepes fixative ½ diluted	36 h	/	X	X		
8a	PBS + 3.5% formaldehyde	36 h	2 h	X			

8b	PBS + 3.5% formaldehyde	36 h	/	X			
9a	4% glutaraldehyde in 0.1 M cacodylate buffer	4 h	2 h (in cacodylate)	X	X	X	7*
9b	4% glutaraldehyde in 0.1 M cacodylate buffer	4 h	/	X	X	X	8*
10a	2% glutaraldehyde + 2% formaldehyde in 0.05 M cacodylate buffer	4 h	2 h (in cacodylate)	X			9*
10 b	2% glutaraldehyde + 2% formaldehyde in 0.05 M cacodylate buffer	4 h	2 h (in PBS)	X	X		
11	Hepes fixative ¼ diluted	36 h	2 h	X	X		
12	Hepes fixative ⅛ diluted	36 h	2 h	X			
13	Hepes fixative with modified osmolarity of 620 mOsm	36 h	2 h	X			
14a	Cacodylatebuffer + 1.5% glutaraldehyde (osm: 275 mOsm)	36 h	2 h	X	X	X	
14b	Cacodylatebuffer + 1,5% glutaraldehyde (osm: 275 mOsm)	36 h	/	X	X		
15a	Cacodylatebuffer + 2% glutaraldehyde + NaCl (osm: 450 mOsm)	36 h	2 h	X	X	X	
15b	Cacodylatebuffer + 2% glutaraldehyde + NaCl (osm: 450 mOsm)	36 h	/	X	X		
16a	Hepes fixative with modified osmolarity of 620 mOsm + 2% formaldehyde	36 h	2 h	X	X	X	
16b	Hepes fixative with modified osmolarity of 620 mOsm + 2% formaldehyde	36 h	/	X	X		
17a	Karnovsky (2% formaldehyde + 2.5% glutaraldehyde in 0.1 M cacodylate buffer)	1 h	1 h (in cacodylate)	X			10
17b	Karnovsky (2% formaldehyde + 2.5% glutaraldehyde in 0.1 M cacodylate buffer)	1 h	2 h (in cacodylate)	X			10
17c	Karnovsky (2% formaldehyde + 2.5% glutaraldehyde in 0.1 M cacodylate)	2.5 h	2 h (in cacodylate)	X			10
18a	4% formaldehyde + 5% glutaraldehyde in 0.1 M cacodylate buffer	1 h	2 h	X	X		11-17

18b	4% formaldehyde + 5% glutaraldehyde in 0.1 M cacodylate buffer	1 h	/	X	X		11*
18c	4% formaldehyde + 5% glutaraldehyde in 0.1 M cacodylate buffer	4 h	2 h	X	X	X	11*
18d	4% formaldehyde + 5% glutaraldehyde in 0.1 M cacodylate buffer	4 h	/	X	X		11*

Protocols from literature that were modified were indicated with 'reference number*'; those that were used unchanged with 'reference number'.

[1]Bian et al. (2010), [2]Mekkawy & Osman (2006), [3]Morrison et al. (1999), [4]Fava & Toledo-Piza (2007), [5]Lønning (1972), [6]Hagström & Lønning (1968), [7]Garcia-Hernandez et al. (2001), [8]Breining & Britz (2000), [9]Hansen & Olafsen (1989), [10]De Spiegelaere et al. (2008), [11]Fausto et al. (1994), [12]Fausto et al. (2004), [13]Scapigliati et al. (1994), [14]Baldacci et al. (2001), [15]Li et al. (2000), [16]Chen et al. (1999), [17]Chen et al. (2007).

Table 4.2 - Overview of adopted buffers and fixatives, the constituents, supplier and preparation protocols. Furthermore, the products used to adjust pH and to perform dehydration series are mentioned.

Buffer/fixative	Total volume	Ingredients	Supplier	Amount	Preparation
Phosphate buffered saline (PBS)	1000 ml	NaCl	Rectorpur, VWR International bvba, Leuven, Belgium	8 g	-Add all ingredients together and add AD until the total volume is 1000 ml
		KH_2PO_4	Merck Chemicals N.V., Overijse, Belgium	0.34 g	
		K_2HPO_4	VWR international bvba, Leuven, Belgium	1.21 g	
		Aquadest (AD)	Silex 2B ST16, Eurowater, Gent, Belgium		
HEPES fixative	500 ml	HEPES fix base*		100 ml	-Heat 15 ml AD until 60 °C
		glutaraldehyde (50%)	Merck Chemicals N.V., Overijse, Belgium	25.28 ml	-Add paraformaldehyde and stirr
		Paraformaldehyde	VWR International bvba, Leuven, Belgium	10 g	-Add 2-5drops of NaOH (2 M) to get all the paraformaldehyde in suspension
		Aquadest (AD)	Silex 2B ST16, Eurowater, Gent, Belgium		-Add HEPES fix base and cool till room temperature
		NaOH (2M)	Merck Chemicals N.V., Overijse, Belgium		-Add glutaraldehyde
					-Adjust pH until 7.2 and add AD until the total volume is 500 ml
*HEPES fix base	500 ml	NaCl	Rectorpur, VWR International bvba, Leuven, Belgium	10.22 g	-Add all ingredients to 400 ml AD and stirr until everything is in suspension

		HEPES (free acid)	Amresco, Solon, USA	17.85 g	-Adjust pH until 7.2 and add AD until the total volume is 500 ml
		$CaCl_2.2H_2O$	Merck Chemicals N.V., Overijse, Belgium	0.74 g	
		AD	Silex 2B ST16, Eurowater, Gent, Belgium		
Karnovsky fixative	50 ml	Ultrapure water	Ultra Clean, Eurowater, Gent, Belgium	12.5 ml	-Heat 12.5 ml ultrapure water until 60°C
		Paraformaldehyde	VWR International bvba, Leuven, Belgium	1 g	-Add 1g paraformaldehyde
		Glutaraldehyde (50%)	Merck Chemicals N.V., Overijse, Belgium	2.5 ml	-Stirr and add a couple of dripps NaOH (2M) to bring everything in solution
		Cacodylate buffer (0.2 M)			-Add 2.5 ml 50% glutaraldehyde
		$CaCl_2$	Merck Chemicals N.V., Overijse, Belgium	0.25 g	-Add cacodylate buffer (0.2M pH 7.4) until 50 ml
		NaOH	Merck Chemicals N.V., Overijse, Belgium		-Add 0.25g $CaCl_2$
Cacodylate buffer (0.2 M)	100 ml	$Na(CH_3)2AsO_2.3H_2O$	EMS, Hatfield, UK	4.28 g	-Dissolve Na-cacodylate in 80 ml ultrapure water
		ultrapure water	Ultra Clean, Eurowater, Gent, Belgium		-Adjust the pH to 7.4 -Add AD until the volume comprises 100 ml.
Hepes fixative with modified osmolarity of 620 mOsm					

HEPES fixative	500 ml	HEPES fix base*		100 ml	-Heat 15 ml AD until 60°C
		glutaraldehyde (50%)	Merck Chemicals N.V., Overijse, Belgium	30 ml	-Add paraformaldehyde and stirr
		Paraformaldehyde	VWR International bvba, Leuven, Belgium	10 g	-Add 2-5drops of NaOH (2 M) to get all the paraformaldehyde in suspension
		Aquadest (AD)	Silex 2B ST16, Eurowater, Gent, Belgium		-Add HEPES fix base and cool till room temperature
		NaOH (2 M)	Merck Chemicals N.V., Overijse, Belgium		-Add glutaraldehyde
					-Adjust pH until 7.2 and add AD until the total volume is 500 ml
*HEPES fix base	2000 ml	NaCl	Rectorpur, VWR International bvba, Leuven, Belgium	2 g	-Add all ingredients to 180 ml AD and stirr until everything is in suspension
		HEPES (free acid)	Amresco, Solon, USA	7 g	-Adjust pH until 7.2 and add AD until the total volume is 2000 ml
		$CaCl_2.2H_2O$	Merck Chemicals N.V., Overijse, Belgium	0.3 g	
		Glutaraldehyde	Merck Chemicals N.V., Overijse, Belgium	1.5%	
Cacodylate buffered glutaraldehyde fixative with modified osmolarity of 275 mOsm	100 ml	$Na(CH_3)2AsO_2.3H_2O$	EMS, Hatfield, UK	2.14 g	-Dissolve Na-cacodylate in 50 ml ultrapure water

		Paraformaldehyde	VWR International bvba, Leuven, Belgium	2 g	-Heat 20 ml AD until 60°C
		Glutaraldehyde (50%)	Merck Chemicals N.V., Overijse, Belgium	3 ml	-Add paraformaldehyde and stirr
		Aquadest (AD)	Silex 2B ST16, Eurowater, Gent, Belgium		-Add 2-5drops of NaOH (2 M) to get all the paraformaldehyde in suspension -Add glutaraldehyde -Adjust pH until 7.2 and add AD until the total volume is 100 ml
Cacodylate buffered glutaraldehyde fixative with modified osmolarity of 450 mOsm	100 ml	$Na(CH_3)2AsO_2.3H_2O$	EMS, Hatfield, UK	2.14 g	-Dissolve Na-cacodylate in 50 ml ultrapure water
		Paraformaldehyde	VWR International bvba, Leuven, Belgium	2 g	-Heat 20 ml AD until 60°C
		Glutaraldehyde (50%)	Merck Chemicals N.V., Overijse, Belgium	4 ml	-Add paraformaldehyde and stirr
		Aquadest (AD)	Silex 2B ST16, Eurowater, Gent, Belgium		-Add 2-5drops of NaOH (2 M) to get all the paraformaldehyde in suspension
		NaCl	Rectorpur, VWR International bvba, Leuven, Belgium	0.5 g	-Add glutaraldehyde and NaCl -Adjust pH until 7.2 and add AD until the total volume is 100 ml
All pH adjustements					

HCl (1 M)	HCl	1mol/l, VWR international bvba, Leuven, Belgium	
NaOH (2 M)	NaOH	Merck Chemicals N.V., Overijse, Belgium	8 g -Dissolve 8 g NaOH in 100 ml AD
	AD	Silex 2B ST16, Eurowater, Gent, Belgium	100ml
Dehydration			
ethanol	Ethanol absolute	AnalaR NORMAPUR, VWR International bvba, Leuven, Belgium	-Dissolve ethanol in AD -SEM: Dehydration is performed in a graded series (10-20-30-40-50-60-70-80-90-100-100%), for each step 7-10 minutes.
acetone	acetone p.	Chem-lab NV, Zedelgem, Belgium	-Dissolve aceton in ethanol -SEM: Dehydration is performed in a graded series (10-20-30-40-50-60-70-80-90-100-100%), for each step 7-10 minutes.

Table 4.3 - Overview of the used fixation and embedding protocols.

	Protocol 1 (modified lab protocol)	Protocol 2 (adapted from Huysseune & Sire (1992))	Protocol 3 (modified lab protocol for chemical fixation of nematodes)
DAY 1	- Fixation (4% glutaraldehyde in 0.1 M cacodylate buffer, minimum 4 h) followed by rinsing with 0.1M cacodylate buffer (3x)	- Fixation (4% glutaraldehyde in 0.1 M cacodylate buffer, minimum 4 h) followed by rinsing with 0.1M cacodylate buffer (3x)	- Fixation (4% glutaraldehyde in 0.1 M cacodylate buffer, minimum 4 h), puncturing during first minutes of fixation - Changing fixative after minimum 4 h - Fridge overnight (rotation)
DAY 2	- Postfixation (1% osmium tetroxide in 0.1 M cacodylate buffer, 2 h) followed by rinsing with 0.1M cacodylate buffer (3x) - Dehydration (graded series of ethanol (Table A.2) followed by graded series of aceton (Table A.2) (7-10 min for each step)) - 1:3 SPURR/ aceton (overnight)	- Postfixation (1% osmium tetroxide in 0.1 M cacodylate buffer, 2 h) followed by rinsing with 0.1M cacodylate buffer (3x) - Puncturing with glass capillary - Dehydration (graded series of ethanol (Table A.2), 7-10 min for each step) - Alcohol/propylene oxide : 10 min - Propylene oxide (2x): 15 min - Propylene oxide/Epon + DMP 30 (1/1)(Epon was added in small amounts): minimum 48 h	- Rinsing with 0.1M cacodylate buffer: o First washing: 15 min o Second washing: 1 h o Third washing: 4 h o Fourth washing - Fridge overnight
DAY 3	- 1:1 SPURR/ aceton (9 h) - 3:1 SPURR/ aceton (overnight)		- Post-fixation (2% osmium tetroxide in 0.1 M cacodylate buffer, 2 h) followed by rinsing with 0.1M cacodylate buffer (3x15 min)

			- Dehydration (graded series of ethanol (Table A.2), 20 min for each step) - Propylene oxide /ethanol 100 (1/1) (15 min) - Propylene oxide pure: 15 min - SPURR/propylene oxide 1:3 (1 h, rotation) - SPURR/propylene oxide 1:1 (overnight fridge, rotation)
DAY 4	- 100% SPURR (9 h) - 100% SPURR (overnight)	- End of the day: Allow evaporation of the propylene oxide	- SPURR/propylene oxide 3:1 (4 h) - SPURR pure (4 h fridge, rotation) - SPURR pure (overnight fridge, rotation)
DAY 5	- Final mould with new SPURR - Polymerization for minimum 9h at 70°C	- Changing Epon - 4h vacuum oven - Final mould with new Epon - Polymerization for minimum 48 h at 60°C	- SPURR pure (fridge, rotation)
DAY 6			- SPURR pure (fridge, rotation)
DAY 7			- SPURR pure (fridge, rotation)
DAY 8			- Final mould with new SPURR - Polymerization for 12 h at 65°C

Table 4.4 - Overview of measurements on 3 different layers of the egg envelope. One-way ANOVA analyses were performed to compare the thickness of each layer between different days of embryonic development. No significant differences ($p<0.05$) were observed between the different days. DPF= days post fertilisation

		Envelope thickness (nm)		
Sampling day	**Replicate (egg)**	**layer 1**	**layer 2**	**layer 3**
1DPF	1	116.47	122.83	8594.99
	2	110.62	88.31	8973.66
	3	153.05	135.72	8882.68
2DPF	1	115.81	104.89	8631.98
	2	100.32	98.13	8191.32
	3	132.22	119.05	10087.86
3DPF	1	94.54	86.45	8624.31
	2	121.87	119.21	8302.35
	3	97.94	92.5	8389.78
4DPF	1	99.42	101.74	7919.41
	2	110.06	144.36	7860.42
	3	105.35	94.99	8273.02
ANOVA p-values		p=0.35	p=0.78	p=0.20

4.7 REFERENCES

Arukwe, A. & Goksoyr, A. (2003) Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. *Comparative hepatology*, **2**, 4.

Arukwe, A., Knudsen, F.R. & Goksoyr, A. (1997) Fish zona radiata (eggshell) protein: A sensitive biomarker for environmental estrogens. *Environmental Health Perspectives*, 105, 418-422.

Baldacci, A.T., A.R.; Mazzini, M.; Fausto, A.M.; Buonocore, F. & Scapigliati, G. (2001) Ultrastructure and proteins of the egg chorion of the antarctic fish *Chionodraco hamatus* (Teleostei, Notothenioidei). *Polar Biology*, **24**, 417-421.

Berois, N., Arezo, M.J. & Papa, N.G. (2011) Gamete interactions in teleost fish: the egg envelope. Basic studies and perspectives as environmental biomonitor. *Biological Research*, **44**, 119-124.

Bian, X.D., Zhang, X.M., Gao, T.X., Wan, R.J., Chen, S.Q. & Sakurai, Y. (2010) Morphology of unfertilized mature and fertilized developing marine pelagic eggs in four types of multiple spawning flounders. *Ichthyological Research*, **57**, 343-357.

Boglione, C., Gavaia, P., Koumoundouros, G., Gisbert, E., Moren, M., Fontagne, S. & Witten, P.E. (2013) Skeletal anomalies in reared European fish larvae and juveniles. Part 1: normal and anomalous skeletogenic processes. *Reviews in Aquaculture*, **5**, S99-S120.

Boglione, C., Gisbert, E., Gavaia, P., Witten, P.E., Moren, M., Fontagne, S. & Koumoundouros, G. (2013) Skeletal anomalies in reared European fish larvae and juveniles. Part 2: main typologies, occurrences and causative factors. *Reviews in Aquaculture*, **5**, S121-S167.

Bohl, R.J., Henry, T.B. & Strange, R.J. (2010) Electroshock-induced mortality in freshwater fish embryos increases with embryo diameter: a model based on results from 10 species. *Journal of Fish Biology*, **76**, 975-986.

Breining, T. & Britz, R. (2000) Egg surface structure of three clingfish species, using scanning electron microscopy. *Journal of Fish Biology*, **56**, 1129-1137.

Chen, C.H., Wu, C.C., Shao, K.T. & Yang, J.S. (2007) Chorion microstructure for identifying five fish eggs of Apogonidae. *Journal of Fish Biology*, **71**, 913-919.

Chen, K.C, Shao, K.T. & Yang, J.S. (1999) Using micropylar ultrastructure for species identification and phylogenetic inference among four species of Sparidae. *Journal of Fish Biology*, **55**, 288-300.

Cheung, W.W.L., Pitcher, T.J. & Pauly, D. (2005) A fuzzy logic expert system to estimate intrinsic extinction vulnerabilities of marine fishes to fishing. *Biological Conservation*, **124**, 97-111.

De Spiegelaere, W., Casteleyn, C., Van Den Broeck, W. & Simoens, P. (2008) Electron Microscopic Study of the Porcine Choroid Plexus Epithelium. *Anatomia Histologia Embryologia*, **37**, 458-463.

Devauchelle, N., Alexandre, J.C., Lecorre, N. & Letty, Y. (1987) Spawning of Sole (*Solea Solea*) in Captivity. *Aquaculture*, **66**, 125-147.

Dwyer, W.P., Fredenberg, W. & Erdahl, D.A. (1993) Influence of electroshock and mechanical shock on survival of trout eggs. *North American Journal of Fisheries Management*, **13**, 839-843.

FAO (2014) The State of World Fisheries and Aquaculture. Rome, 223.

Faustino, F., Nakaghi, L.S.O., Marques, C., Ganeco, L.N. & Makino, L.C. (2010) Structural and ultrastructural characterization of the embryonic development of *Pseudoplatystoma* spp. hybrids. *International Journal of Developmental Biology*, **54**, 723-730.

Fausto, A.M., Carcupino, M., Scapigliati, G., Taddei, A.R. & Mazzini, M. (1994) Fine-Structure of the Chorion and Micropyle of the Sea Bass Egg *Dicentrarchus Labrax* (Teleostea, Percichthydae). *Bollettino Di Zoologia*, **61**, 129-133.

Fausto, A.M., Picchietti, S., Taddei, A.R., Zeni, C., Scapigliati, G., Mazzini, M. & Abelli, L. (2004) Formation of the egg envelope of a teleost, *Dicentrarchus labrax* (L.): immunochemical and cytochemical detection of multiple components. *Italian Journal of Anatomy and Embryology*, **208**, 43-53.

Fava, D. & Toledo-Piza, M. (2007) Egg surface structure in the annual fishes *Simpsonichthys* (subgenera Ophthalmolebias and Xenurolebias) and *Nematolebias* (Teleostei : Cyprinodontiformes : Rivulidae): variability and phylogenetic significance. *Journal of Fish Biology*, **71,** 889-907.

Godfrey, H. (1957) Mortalities among Developing Trout and Salmon Ova Following Shock by Direct-Current Electrical Fishing Gear. *Journal of the Fisheries Research Board of Canada*, **14**, 153-164.

Gwo, H.-H. (2008) Morphology of the fertilizable mature egg in the *Acanthopagrus latus*, A. schlegeli and *Sparus sarba* (Teleostei: perciformes: sparidae). *Journal of Microscopy*, **232**, 442-452.

Hagstrom, B.E. & Lønning, S. (1968) Electron Microscopic Studies of Unfertilized and Fertilized Eggs from Marine Teleosts. *Sarsia*, **33**, 73-80.

Hansen, G.H. & Olafsen, J.A. (1999) Bacterial interactions in early life stages of marine cold water fish. *Microbial Ecology*, **38**, 1-26.

Henry, T.B. & Grizzle, J.M. (2004) Survival of largemouth bass, bluegill and channel catfish embryos after electroshocking. *Journal of Fish Biology*, **64**, 1206-1216.

Hernandez, M.P.G., Lozano, M.T., Elbal, M.T. & Agulleiro, B. (2001) Development of the digestive tract of sea bass (*Dicentrarchus labrax* L). Light and electron microscopic studies. *Italian Journal of Anatomy and Embryology*, **204**, 39-57.

Hirai, A. (1988) Fine structures of the micropyles of pelagic eggs of some marine fishes. *Japanese Journal of Ichthyology*, **35**, 351-357.

Huysseune, A. & Sire, J.Y. (1992) Development of Cartilage and Bone Tissues of the Anterior Part of the Mandible in Cichlid Fish - a Light and Tem Study. *Anatomical Record*, **233**, 357-375.

Iconomidou, V.A., Chryssikos, D.G., Gionis, V., Pavlidis, M.A., Paipetis, A. & Hamodrakas, S.J. (2000) Secondary structure of chorion proteins of the teleostean fish *Dentex dentex* by ATR FT-IR and FT-Raman spectroscopy. *Journal of Structural Biology*, **132**, 112-122.

Imsland, A.K., Foss, A., Conceicao, L.E.C., Dinis, M.T., Delbare, D., Schram, E., Kamstra, A., Rema, P. & White, P. (2003) A review of the culture potential of *Solea solea* and *S. senegalensis*. *Reviews in Fish Biology and Fisheries*, **13**, 379-407.

Iwamatsu, T., Ishijima, S. & Nakashima, S. (1993) Movement of Spermatozoa and Changes in Micropyles during Fertilization in Medaka Eggs. *Journal of Experimental Zoology*, **266**, 57-64.

Iwamatsu, T. & Ohta, T. (1981) Scanning Electron-Microscopic Observations on Sperm Penetration in Teleostean Fish. *Journal of Experimental Zoology*, **218**, 261-277.

Iwamatsu, T.O., K., Yoshimoto, Y. & Hiramoto, Y. (1991) Time sequence of early events in fertilization in the Medaka egg. *Development Growth & Differentiation*, **33**, 479-490.

Jeon, K.W. & Friedlander, M. (1992) *International review of cytology*, 1st edition, Academic Press, US, **136**, 342p.

Kobayashi, W. & Yamamoto, T.S. (1981) Fine-Structure of the Micropylar Apparatus of the Chum Salmon Egg, with a Discussion of the Mechanism for Blocking Polyspermy. *Journal of Experimental Zoology*, **217**, 265-275.

Li, Y., Wu, C. & Yang, J. (2000) Comparative ultrastructural studies of the zona radiata of marine fish eggs in three genera in Perciformes. *Journal of Fish Biology*, **56**, 615-621.

Lønning, S. & Davenport, J. (1980) The Swelling Egg of the Long Rough Dab, *Hippoglossoides-platessoides Limandoides* (Bloch). *Journal of Fish Biology*, **17**, 359-378.

Lønning, S., Kjorsvik, E. & Falkpetersen, I.B. (1988) A Comparative-Study of Pelagic and Demersal Eggs from Common Marine Fishes in Northern Norway. *Sarsia,* **73**, 49-60.

Lønning, S. (1972) Comparative electron – Microscopic studies of teleostean eggs with special reference to chorion. *Sarsia*, **49**, 41-48.

Mekkawy, I.A.A. & Osman, A.G.M. (2006) Ultrastructural studies of the morphological variations of the egg surface and envelopes of the African catfish *Clarias gariepinus* (Burchell, 1822) before and after fertilisation, with a discussion of the fertilisation mechanism. *Recent advances in the study of fish eggs and larvae*, 23-40.

Morrison, C., Bird, C., O'neil, D., Leggiadro, C., Martin-Robichaud, D., Rommens, M. & Waiwood, K. (1999) Structure of the egg envelope of the haddock, *Melanogrammus aeglefinus*, and effects of microbial colonization during incubation. *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, **77**, 890-901.

Muth, R.T. & Ruppert, J.B. (1997) Effects of electrofishing fields on captive embryos and larvae of razorback sucker. *North American Journal of Fisheries Management*, **17**, 160-166.

Oppenberntsen, D.O., Helvik, J.V. & Walther, B.T. (1990) The Major Structural Proteins of Cod (*Gadus Morhua*) Eggshells and Protein Crosslinking during Teleost Egg Hardening. *Developmental Biology*, **137**, 258-265.

Ravaglia, M.A. & Maggese, M.C. (2003) Ovarian follicle ultrastructure in the teleost *Synbranchus marmoratus* (Bloch, 1795), with special reference to the vitelline envelope development. *Tissue and Cell,* **35**, 9-17.

Riehl, R. (1980) Micropyle of Some Salmonins and Coregonins. *Environmental Biology of Fishes*, **5**, 59-66.

Riehl, R. & Kokoscha, M. (1993) A Unique Surface Pattern and Micropylar Apparatus in the Eggs of *Luciocephalus* Sp (Perciformes, Luciocephalidae). *Journal of Fish Biology*, **43**, 617-620.

Rizzo, E., Sato, Y., Barreto, B.P. & Godinho, H.P. (2002) Adhesiveness and surface patterns of eggs in neotropical freshwater teleosts. *Journal of Fish Biology*, **61**, 615-632.

Scapigliati, G., Carcupino, M., Taddei, A.R. & Mazzini, M. (1994) Characterization of the Main Egg Envelope Proteins of the Sea Bass *Dicentrarchus Labrax* L (Teleostea, Serranidae). *Molecular Reproduction and Development*, **38**, 48-53.

Scapigliati, G., Fausto, A.M., Zega, S. & Mazzini, M. (1995) Characterization of the Main Egg Chorion Proteins of the Whitefish *Coregonus Lavaretus* L (Osteichthyes, Salmonidae). *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*, **112**, 169-175.

Soetaert, M., Decostere, A., Polet, H., Verschueren, B. & Chiers, K. (2013) Electrotrawling: a promising alternative fishing technique warranting further exploration. *Fish and Fisheries*, **16**, 104-124.

Stehr, C.M. & Hawkes, J.W. (1979) Comparative Ultrastructure of the Egg Membrane and Associated Pore Structures in the Starry Flounder, *Platichthys Stellatus* (Pallas), and Pink Salmon, *Oncorhynchus Gorbuscha* (Walbaum). *Cell and Tissue Research*, **202**, 347-356.

Thurstan, R.H., Brockington, S. & Roberts, C.M. (2010) The effects of 118 years of industrial fishing on UK bottom trawl fisheries. *Nature Communications*, **1**.

Vancoppenolle, B., Claeys, M., Borgonie, G., Tytgat, T. & Coomans, A. (2000) Evaluation of fixation methods for ultrastructural study of *Caenorhabditis elegans* embryos. *Microscopy Research and Technique*, **49**, 212-216.

Chapter 5 DEVELOPMENT OF A RELIABLE EXPERIMENTAL SET-UP FOR DOVER SOLE LARVAE *SOLEA SOLEA* AND EXPLORING THE POSSIBILITY OF IMPLEMENTING THIS HOUSING SYSTEM IN A GNOTOBIOTIC MODEL

Based on: De Swaef, E., Demeestere, K., Boon, N., Van den Broeck, W. Haesebrouck, F. & Decostere, A. (2017) Development of a reliable experimental set-up for Dover sole larvae *Solea solea* L. and exploring the possibility of implementing this housing system in a gnotobiotic model. *Research in Veterinary Science*, **115**, 418-424.

5.1 ABSTRACT

Due to the increasing importance of the aquaculture sector, diversification in the number of cultured species imposes itself. Dover sole (*Solea solea)* is put forward as an important new aquaculture candidate due to its high market value and high flesh quality. However, as for many other fish species, sole production is hampered by amongst others high susceptibility to diseases and larval mortality, rendering the need for more research in this area. In this respect, in first instance, a housing system for Dover sole larvae was pinpointed by keeping the animals individually in 24-well plates for 26 days with good survival rates and initiating metamorphosis. This ensures a standardised and reliable experimental set-up in which the possible death of one larva has no effect on the other larvae, rendering experiments adopting such a system more reproducible. In addition to proving valuable in many other applications, this multi well system constitutes a firm basis to enable the gnotobiotic rearing of larvae, which hitherto is non-existing for Dover sole. In this respect, secondly, a large number of disinfection protocols was tested, making use of widely employed disinfectants as hydrogen peroxide, glutaraldehyde and/or ozone whether or not combined with a mixture of antimicrobial agents for 24h. Although none of the tested protocols was sufficient to reproducibly generate a gnotobiotic model, the combination of glutaraldehyde and hydrogen peroxide resulted in hatchable, bacteria-free eggs in some cases.

5.2 INTRODUCTION

Mariculture in the European Union is dominated by three species (salmon (*Salmo salar)*, sea bass (*Dicentrachus labrax)* and gilthead seabream (*Sparus aurata))* representing 27% of farmed fish (European Commission, 2016). Further expansion of the European aquaculture sector is only possible by overcoming the production bottlenecks, promoting the consumption of fish cultured in Europe and diversifying the industry by including new fish species (European Union DIVERSIFY, 2016; Lane et al., 2014). Dover sole (*Solea solea)* is an economically interesting and potentially important aquaculture candidate due to its high market value and flavourful flesh (Ferraresso et al., 2013). However, as for many other fish species, sole production is hampered by high susceptibility to diseases, juvenile mortality (Ferraresso et al., 2013) and insufficient knowledge of the nutritional requirements (Cahu and Infante, 2001). In addition, difficulties in larval rearing are a marked hindrance, which is reflected in low and unpredictable survival and slow growth (Boglione et al., 2013a, b). The latter warrants research on the larval production phase as an aid in promoting flatfish aquaculture. To enable this, standardized and controlled experimental set-ups are a necessary prerequisite. The classical set-up encompasses larvae being group-housed in tanks or cylinders. However, this way of housing may result in large variations between replicates (Bolle et al., 2012; Situmorang et al., 2014) thereby hampering experimental reproducibility. This may at least partially be explained by the death of one larva impacting the health status of the other larvae housed in the same water body. Indeed, the presence of decomposing dead larvae results in decreased oxygen and increased ammonia levels, thereby jeopardizing larval survival (Goncalves & Gagnon, 2011; Wang et al., 2015). Furthermore, changes in virulence of a pathogenic agent due to variable amounts of (sterile) dead larvae present in the rearing vial were reported (Li et al., 2014), which may greatly influence the reproducibility of pathogenesis studies. The above listed disadvantages may be tackled by housing larvae individually in multi well plates. Implementing a well plate system also facilitates monitoring of the health status and behaviour of the individually housed larvae. These parameters, in addition to mortality, may be adopted to assess the impact of a specific treatment. Individual wells are considered independent replicates for statistical analysis (OECD, 2013) enabling a high number of replicates with only one larva per well thereby reducing the number of experimental animals needed. Although the multi well plate housing system was already developed for several species (Panini et al., 2001; Sandlund et al., 2010; Forberg et al., 2011; Martin et al., 2014; Schaeck et al., 2016) and used successfully in efficacy studies involving probiotics (Makridis et al., 2000; 2001; Planas et al., 2006) and immunostimulants (Skjermo & Bergh, 2004), no information on the practicability of multi well plates as an experimental set-up for Dover sole larvae is available.

Furthermore, a multi well system was already implemented in different gnotobiotic experimental systems, facilitating the housing of the bacteria-free larvae (Forberg et al., 2011; Schaeck et al., 2016). Gnotobiotic models are an excellent tool to investigate interactions between bacteria and the host. These models require not only a reduction of the bacterial load but a complete elimination of (possibly pathogenic) bacteria also needs to be achieved by egg disinfection. However, no data on the toxicity nor efficacy of disinfectants for Dover sole eggs are available. For the closely related Senegalese sole (*Solea senegalensis*), only iodine was evaluated (Martin et al., 2014).

In this respect, the aim of this study was twofold. First and foremost, we endeavoured to establish a multi well housing system as a means to facilitate larval research on Dover sole. Secondly, we attempted to use this experimental set-up for creating a gnotobiotic model by further exploring the disinfection possibilities for Dover sole eggs and identifying an effective and safe disinfection protocol, that is with no impact on egg viability and hatching but resulting in a complete elimination of the bacteria.

5.3 MATERIALS AND METHODS

5.3.1 Pinpointing a reliable experimental set-up to house Dover sole larvae in 24-well plates

Solea solea eggs were obtained from the Institute for Marine Resources and Ecosystem Studies (IMARES) in IJmuiden, the Netherlands. Eggs were naturally spawned overnight and collected the next morning. The dead eggs were removed, whereafter transportation to the research facilities in natural seawater (32 gL^{-1}) occurred. Upon arrival, eggs were acclimatized with artificial seawater (ASW) of 34 gL^{-1} (Instant Ocean, Aquarium Systems, Mentor, Ohio) and further incubated herein under aeration.

One day after arrival, dead eggs were removed and developing eggs were put on a nylon sieve (mesh size 150 µm) and submerged in ASW with 1% hydrogen peroxide (H_2O_2, 30 % solution in water, Sigma Aldrich, Steinheim, Germany) for 3 minutes. After disinfection, eggs were rinsed and kept in 400 mL ASW in glass bottles at 16 ± 1°C. These bottles were aerated with filtered (0.2 µm, Sartorius AG, Göttingen, Germany) sterile air. Two days after hatching (DAH), larvae were placed individually in sterile polystyrene 24-well tissue culture plates (Greiner Bio-One, Frickenhausen, Germany), each well containing approximately 2 mL ASW. Translocation of the newly hatched larvae was performed one by one by means of a 3 mL graduated plastic Pasteur pipette (VWR, Leuven, Belgium) with cut-off point to avoid damage to the entering larvae. The wells were incubated at 16 ± 1°C and the larvae were fed ad libitum with sterile live *Artemia franciscana* nauplii (EG type; INVE Aquaculture NV, Belgium) every other day, starting from 6 DAH onwards. Sterile *Artemia* cysts and nauplii were obtained through decapsulation (Sorgeloos, 1986). Half of the well water was replaced every other day and the larvae were monitored until 26 DAH. The presence of *Artemia* in the intestinal tract of the larvae was daily

macroscopically verified. All animals were subjected to a circadian rhythm of 9 hours light and 15 hours darkness. The 24-well plates were placed on a dark surface and translocation was restricted to a minimum. This experimental set-up was conducted in quadruplicate with different egg batches each containing 96 larvae divided over four 24-well plates. The appropriate control groups were included, of which the eggs and resulting larvae underwent the same physical handling, without the addition of H_2O_2.

To verify if the 24-well plates allow larval metamorphosis, larvae of four different batches were monitored until 26 DAH and the onset of metamorphosis in the majority of the larvae was stereomicroscopically confirmed focusing on eye migration (Fernandez-Diaz et al., 2001; Pallazzi et al., 2006).

Evaluation of the implemented disinfection protocol was performed by comparing the hatching rate and bacterial load with the control group. Samples for bacteriological analysis were taken immediately following disinfection and were divided in four subsamples of 10-15 eggs which were inoculated in two tubes filled with 5 mL Tryptic soy broth (TSB, Becton, Dickinson and Company, New Jersey, USA) supplemented with 1.5% NaCl, onto Thiosulfate Citrate Bile Sucrose Agar plates (TCBS, Sigma Aldrich, ST Louis, USA) and onto Marine Agar plates (MA, Scharlab S.L., Sentmenat, Spain). Following incubation at 17 ± 1°C for at least 3 weeks, bacterial growth on agar plates was expressed using a semi-quantitative score: 3 (>100 colonies/plate), 2 (10-100 colonies/plate), 1 (1-10 colonies/plate) or 0 (no bacterial growth). Turbidity of the TSB (+1.5% NaCl) was considered to point towards the presence of multiplying bacteria.

5.3.2 Evaluation of disinfection protocols to generate a gnotobiotic model

All manipulations were performed within a microbiological safety cabinet class II. All materials and liquids were autoclaved (120°C, 20 minutes) or purchased sterile. Artificial autoclaved seawater (AASW; Instant Ocean®) was adjusted to a salinity of 34 gL^{-1} and a temperature of 16 ± 1°C. Three different disinfecting agents were tested, either single or combined: glutaraldehyde (Salvesen et al., 1997; Dierckens et al., 2009), hydrogen peroxide (Douillet & Holt, 1994; Verner-Jeffreys et al., 2007) and ozone (Douillet & Holt, 1994; Ben-Atia et al., 2007). The various protocols are listed in Table 5.1.

Prior to disinfection, eggs were collected on a nylon fish net and one gram of eggs per replicate (corresponding with approximately 600 eggs) was weighed. Next, the eggs were put on a nylon sieve (mesh size 150 µm) and submerged in AASW with glutaraldehyde (50 wt% solution in water, Merck, Hohenbrunn, Germany) or hydrogen peroxide (30 % solution in water, Sigma Aldrich, Steinheim, Germany) with varying concentrations (H_2O_2: 2 to 12%; glutaraldehyde: 400 to 800 ppm) and contact times (H_2O_2: 1 to 5 min; glutaraldehyde: 1 to 10 min) depending on the protocol used (Table 5.1). Each

protocol was replicated two to eight times. During disinfection, the sieve was gently moved to ensure an even distribution of the disinfectant. Subsequently, the eggs were rinsed with 1 L AASW and in case a second disinfection step was performed, the protocol was repeated starting from submersal in the appropriate disinfectant solution.

For the ozone treatment, the eggs were placed in a glass bottle containing 300 mL AASW and supplied with a special glass cap through which ozone produced in dry air by means of a LAB2B ozone generator (Ozonia, Duebendorf, Switzerland), was bubbled during 2 or 4 minutes. To compare different ozone treatments, the multiplication of the ozone concentration and contact time, denoted as "CT", was used.

Depending on the protocol (Table 5.1), eggs were additionally submerged for 24h in AASW supplemented with two or more of the following antimicrobial agents: ampicillin (10 ppm), rifampicin (10 ppm), penicillin (150 ppm), streptomycin (75 ppm), oxolinic acid (10 ppm), kanamycin (10 ppm) and erythromycin (10 ppm) (Munro et al., 1995; Rawls et al., 2004; Verner-Jeffreys et al., 2007; Dierckens et al., 2009; Forberg et al., 2011; Situmorang et al., 2014; Schaeck et al., 2016). All antimicrobial agents were purchased from Sigma Aldrich (Steinheim, Germany). Following 24h of incubation, the eggs were aseptically rinsed with 1 L AASW.

After completing the disinfection protocols, the eggs were aseptically transferred to a 500 mL glass incubation bottle containing 400 mL unsupplemented AASW. The eggs of the control group underwent the same procedures as the disinfected eggs but no disinfectant nor antimicrobial agents were added to the AASW.

The hatching rate for each incubation bottle was expressed using a semi-quantitative score (Schaeck et al., 2016): 3 ('Good', the hatchability is comparable with the eggs in the non-treated control group), 2 ('Moderate', the hatchability is good, but not as high as the non-treated control group), 1 ('low', the hatching rates are very low, compared with the non-treated control group) and 0 ('none', no hatching at all) (Table 5.1).

Depending on the protocol, both culture based techniques as well as a flow cytometer analysis were used to verify a reduction in bacterial load or a complete elimination of bacteria. Bacteriological analysis of the disinfected eggs based on inoculating MA, TCBS and TSB supplemented with 1.5% NaCl was performed as described above. In part of the experiments, a flow cytometer was applied (Schaeck et al., 2016) on water samples of the rearing bottles taken just after disinfection (Table 5.1).

In case the media inoculated with eggs did not exhibit bacterial growth after four days of incubation and live bacteria were not detected by the flow cytometer system, the alleged bacteria-free larvae

resulting from the corresponding bottles were further employed using the previously described housing system for Dover sole eggs. Sole larvae (2 DAH) were housed individually in sterile polystyrene 24-well tissue culture plates (Greiner Bio-One, Frickenhausen, Germany), each well containing 2 mL AASW. The 24-well plates were placed in a barrier isolator with a glove system (G(ISO)-T3, TCPS, Rotselaar, Belgium). Half of the well water was replaced aseptically every other day and larvae were tested at 6 DAH for their bacteria-free status by analysing the rearing water by means of flow cytometry.

5.4 RESULTS

5.4.1 Pinpointing a reliable experimental set-up to house Dover sole larvae in 24-well plates

Disinfection of the sole eggs with 1% H_2O_2 for 3 minutes resulted in a hatching rate similar to the hatching of the control group (semi-quantitative score of 3). For the non-treated control group, bacterial growth on MA and TCBS resulted in a semi-quantitative score of 3. After disinfection, bacterial growth on TCBS was absent and 50% of the MA plates showed a semi-quantitative score of 2. Tryptic soy broth + 1% NaCl was turbid in all cases.

Survival rates for the larvae of four different batches until 26 DAH were 85%, 84%, 65% and 58% respectively.

Metamorphosis was observed in the vast majority of the larvae maintained in the well plates until 26 DAH (Figure 5.1). Starting from feeding, a large amount of *Artemia* nauplii (observable as orange particles) was perceived daily in the intestinal tract of the transparent larvae. It was confirmed that feeding occurred ad libitum, since the well water systematically contained residual *Artemia* nauplii at the next feeding time. The majority of these remaining *Artemia* nauplii were removed during water exchange (just before feeding).

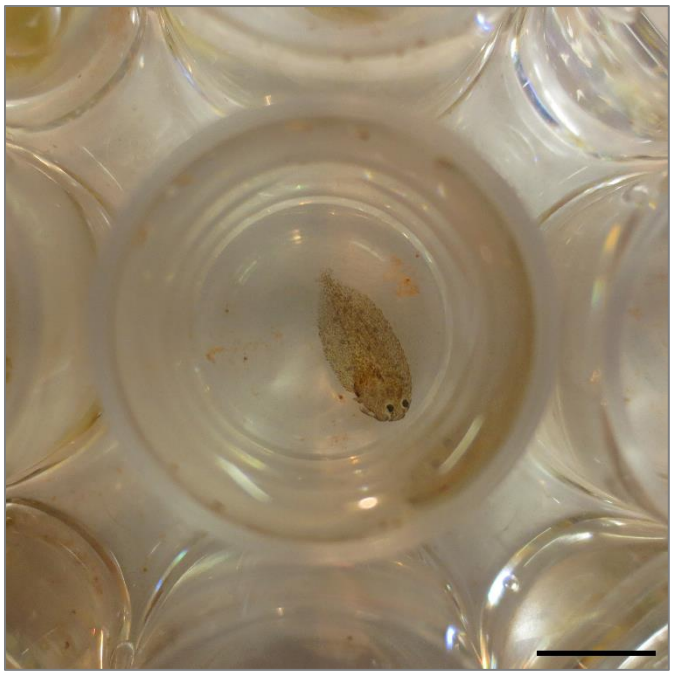


Figure 5.1 - Dover sole larva at 26 days post hatching, housed individually in a 24-well plate showing metamorphosis. Scale bar: 5mm.

5.4.2 Disinfection protocols

Hatching rates of the non-disinfected sole eggs varied greatly between batches. Bacterial growth on agar plates of the control eggs (without disinfection) consistently resulted in the maximum semi-quantitative score of 3. Turbidity of TSB + 1.5% NaCl was observed in all control samples. All results regarding the disinfection experiments are summarized in Table 5.1.

5.4.2.1 Hydrogen peroxide

Low concentrations of hydrogen peroxide (2% H_2O_2) resulted in a semi-quantitative hatching score of 3. Increasing the hydrogen peroxide concentration to 8% or more with the same contact time decreased hatching rates to zero with no marked increase in the number of samples with a reduced bacterial growth. For the higher concentrations (3% or more), lowering the contact time to 1 or 2 minutes did not result in a notably increased hatching. Tryptic soy broth + 1.5% NaCl and MA displayed bacterial growth in almost all tested protocols, but a lower semi-quantitative score for the bacterial growth was noticed in part of the replicates starting from 1% H_2O_2. Concentrations of 2% H_2O_2 or higher completely inhibited the formation of bacterial colonies on TCBS (except one sample). Combining concentrations of 1 or 2% H_2O_2 with an antimicrobial mixture for 24 hours resulted in a reduction of the bacterial growth (semi-quantitative score 1 or 2). However, no culture media without bacterial growth were observed.

5.4.2.2 Glutaraldehyde

Contact times of 2.5 minutes gave the maximum semi-quantitative hatching score of 3 but when increased to four minutes and more, variable hatching results were seen. Overall, a reduction of

bacterial load was noted in most of the replicates and a number of treatments resulted in some replicates without bacterial growth, although inconsistent results were found. When glutaraldehyde (400 ppm, 4 minutes) was combined with antimicrobial agents for 24 hours, three out of eight replicates resulted in no bacterial growth in TSB + 1.5% NaCl, with two of these being bacteria-free assessed by means of flow cytometry. Bacterial growth was observed on all plates inoculated with well water of the resulting larvae (6 DAH). Two rounds of glutaraldehyde (400 ppm, 2.5 or 5 minutes), whether or not combined with antimicrobial agents for 24 hours, resulted in no hatching eggs although a reduction in bacterial growth was found in most of the MA plates (semi-quantitative score of <3) and most of the TCBS plates remained without bacterial growth.

5.4.2.3 Ozone

Different contact times and concentrations were tested but hatching was low to not present if ozone concentrations exceeded 2 mg L_{gas}^{-1}. Combining ozone with antimicrobial agents resulted in no hatching at all. Turbidity of TSB + 1.5% NaCl was observed for all tested concentrations and contact times, apart from CT 12 mg min L_{gas}^{-1} (one out of the two replicates was sterile). A reduction in bacterial growth on MA plates was shown for most of the replicates. TCBS plates contained bacterial colonies in nine out of 20 replicates and a CT value of 8 mg min L_{gas}^{-1} or higher was necessary to generate plates without bacterial growth.

5.4.2.4 Hydrogen peroxide and ozone

For the combination of hydrogen peroxide and ozone, the lowest tested concentration of ozone (2 mg L_{gas}^{-1}) was preceded by applying a low concentration of hydrogen peroxide (1%). Both concentrations separately resulted in moderate to good hatching results. However, combining both disinfectants caused very bad to no hatching at all. Although part of the TCBS plates showed no bacterial growth, all TSB + 1.5% NaCl samples were turbid and only one third of the samples on MA resulted in a semi-quantitative score <3 for bacterial growth.

5.4.2.5 Glutaraldehyde and ozone

Combining glutaraldehyde with ozone disinfection resulted in no surviving larvae. A clear reduction in bacterial growth was observed, considering all TSB + 1.5% NaCl samples displayed no bacterial growth, neither did half of the MA plates nor half of the TCBS plates.

5.4.2.6 Hydrogen peroxide and glutaraldehyde

Hydrogen peroxide followed by glutaraldehyde treatment and an antibiotic mixture, gave good hatching results in most of the protocols. When hydrogen peroxide was used as the first disinfection agent, concentrations varied from 1 to 4% but the contact time remained 3 minutes in all protocols. The subsequent use of glutaraldehyde in concentrations of 400 to 800 ppm with contact times between 1 and 5 minutes resulted in a reduction of the bacterial load in all replicates. No bacterial

growth was observed in the majority of the TSB samples and most of the MA and TCBS plates. The most promising protocols based on high semi-quantitative scores for hatching and large decreases in bacterial growth, were 1% H_2O_2 (3 minutes)*400 ppm glutaraldehyde (2.5 minutes), 1% H_2O_2 (3 minutes)*400 ppm glutaraldehyde (3.5 minutes), 2% H_2O_2 (3 minutes)*400 ppm glutaraldehyde (2.5 minutes), 2% H_2O_2 (3 minutes)*400 ppm glutaraldehyde (3.5 minutes) and 2% H_2O_2 (3 minutes)*400 ppm glutaraldehyde (4 minutes). Consequently, these protocols were tested in six- to tenfold to verify the reproducibility of the protocol. However, none of the protocols seemed to give consistent results concerning both hatchability and sterility (based on a flow cytometry).

Disinfection with 1% H_2O_2 (3 minutes) prefaced with 400 ppm glutaraldehyde resulted in bacterial growth in the TSB + 1.5% NaCl samples when the shortest contact time was used (2.5 minutes). Besides, a reduction in bacterial growth was found in part of the samples of MA and all of TCBS agar plates. Almost no hatching was encountered when the longest contact time was applied (7 minutes).

5.5 DISCUSSION

Bacterial overgrowth in a multi well system, which is a static water body, may lead to larval mortality. This accentuates the need to disinfect the eggs when developing an experimental set-up to house Dover sole larvae. Disinfection with 1% H_2O_2 for 3 minutes did not impair hatching and reduced the bacterial load. In addition, it encompasses an environmentally and user friendly (Kiemer & Black, 1997) protocol with a relatively low cost. The resulting larvae were successfully reared in 24-well plates until 26 DAH in an apparently good health status (incl. good feeding behaviour) and with higher survival rates (between 58 and 85%) compared with small scale experiments (> 50%, Bolle et al., 2012) or hatchery conditions (on average 44%, Palazzi et al., 2006). Furthermore, metamorphosis was initiated in the majority of the larvae. Hatching of the eggs was observed to be remarkably low (data not shown) in the experiment with the lowest larval survival rate, indicating a possible inferior batch quality. Large differences in egg quality during spawning season were indeed observed for many species, including Dover sole (Devauchelle et al., 1987).

Viral infections such as viral encephalopathy and retinopathy were reported in Dover sole adults (Baghesan et al., 2003; Zanella, 2004). However, information on the prevalence in Dover sole larvae is limited. Therefore, testing the virucidal potential of the tested disinfection protocols was not the aim of this study. However, in future experiments, investigating the efficacy of the developed disinfection protocol on the viral load might be important as well.

The start of the feeding regime of Dover sole varies greatly between studies. First feeding may indeed already be started at 3 DAH (Devauchelle et al., 1987; Dinis et al., 1999; Imsland et al., 2003) or 4 DAH (Palazzi et al., 2006; Parma et al., 2013). However, the temperature was higher in the latter studies

(18-20°C) compared with our experiments (16°C). Lower temperatures decrease the metabolic rate and yolk sac absorption in fish larvae (Fonds, 1972; Fukuhara, 1990; Blaxter, 1992; Pepin et al., 1997), which may justify a later starting point of *Artemia* feeding. Furthermore, during the first days of feeding, larvae use a lot of energy by searching and capturing live prey when housed in hatchery conditions or in the open sea. In this period, energy gain is limited or negative (Richard et al., 1991). Housing the larvae in 24-well plates and feeding them in excess in these small wells, markedly reduces the effort for searching and capturing *Artemia*s, making this period of energy depletion much less applicable in our housing system and again supporting the later onset of feeding live prey. In addition, preliminary trials at our department indicated that feeding in an earlier stage (4 DAH) did not result in significant amounts of caught prey in the intestines of the small larvae (data not shown). As a consequence, a large amount of remaining *Artemia* nauplii needed to be removed from the wells, which may result in increased handling stress for the animals. Starting the feeding regime at 6 DAH, resulted in adequate feeding behaviour of the larvae starting from the first feeding day without compromised metamorphosis or increased mortality until 26 DAH.

In several studies, fish larvae were housed in 96-well plates in order to determine hatching rates, either to compare treatments or to evaluate egg quality (Panini et al., 2001; Felip et al., 2009; Forberg et al., 2011). Larvae were only kept in these wells until hatching was completed. In our study, 24-well plates were employed since the larvae were kept for a longer period of time and therefore needed a larger water body to favour adequate water quality and space. The well plates were placed on a dark surface to ensure a good distinction by the larvae between the bottom of the well and the water surface. Movement of the multi well plates was restricted to a minimum, to limit the concomitant stress.

Housing the larvae individually in well plates provides the major advantage of being able to monitor the behaviour and condition of each animal separately. Including these parameters in addition to mortality assessment may be essential in studies concerning the effect of toxins, drugs or genetic mutations on the nervous system (Gosselin et al., 1989; Samson et al., 2008; Norton, 2013). Behavioural monitoring may be done with automated observation systems using video tracking as already frequently used for behavioural research involving zebrafish (Ahmad et al., 2012).

In the framework of animal ethics, the use of this experimental setup may contribute to both ‘Reduction’ and ‘Refinement’ (Russell and Burch, 1959) in scientific experiments involving fish larvae. Since individual wells are considered independent replicates (OECD, 2013), less animals are needed to assure statistically robust experiments. Including sublethal parameters to determine the extent to which the fish larvae are experiencing pain, distress and harm, may assist in refining experiments and hence increasing welfare. Pain indicators and humane endpoints are well delineated for rabbits and

rodents (Kohn et al., 2007). Also strong indications for both pain awareness or suffering in fish have been published (Sneddon, 2003; 2009; 2015; Braithwaite & Huntingford, 2004; Brown, 2015) and objectively observable indicators for pain are proposed for adult fish (reviewed in Sneddon, 2015). However, research concerning larval pain virtually is non-existent. Consequently, no specific features nor behavioural parameters are available to evaluate larval welfare and, in a next step, identify humane endpoints. This experimental model may facilitate research in this domain with the individual housing of the larvae as a major asset, hereby enabling individual behavioural analyses.

Only low concentrations of the various tested disinfectants were necessary to abrogate bacterial growth on TCBS agar, including the 1% H_2O_2 for 3 minutes disinfection protocol, as used in the multi well housing system. TCBS agar is considered to be a selective medium commonly used to isolate *Vibrio* species (West et al., 1982; Pfeffer & Oliver, 2003; Williams et al., 2013; Interaminense et al., 2014). Vibrios may be associated with larval mortality in various fish species, resulting in major economic losses to the aquaculture industry (Ishimaru et al., 1996; Gatesoupe et al., 1999; Villamil et al., 2003; Thomson et al., 2005; Reid et al., 2009). However, one should make an aside on the selectivity of the medium which is questioned as various bacterial species not belonging to the *Vibrio* genus may grow on TCBS (Shikongo-Nambabi et al., 2010; Trang, 2014), including *Staphylococcus*, *Flavobacterium*, *Shewanella* and *Pseudoaltermonas* spp. (Thompson et al., 2004).

Hydrogen peroxide, glutaraldehyde and ozone are well-known for their disinfecting properties and hence frequently used as fish egg disinfectants. It is common knowledge that the tolerance to disinfectants is very species dependent, impeding extrapolation of a protocol across species (reviewed in De Swaef et al., 2015). Hydrogen peroxide has already been used as an egg disinfectant for many different fish species, reducing the bacterial load in the vast majority of cases (reviewed in De Swaef et al., 2015). Bacteria-free red drum (*Sciaenops ocellatus*) eggs were obtained after disinfection with 3% H_2O_2 for 5 minutes (Douillet & Holt, 1994). When concentrations of 3% were adopted for Dover sole egg disinfection, hatching decreased significantly, even after short contact times, indicating a higher sensitivity of this species to H_2O_2. Glutaraldehyde is widely used as egg disinfectant for various species in many different concentrations and contact times (reviewed in De Swaef et al., 2015). A single dose of glutaraldehyde resulted in part of the eggs being sterile in three different studies (Salvesen et al., 1997; Morehead & Hart, 2003; Can et al., 2010). Applying one or two treatments with glutaraldehyde whether or not followed by a single or continuous administration of an antibiotic mixture, generated a gnotobiotic model for sea bass (Dierckens et al., 2009; Schaeck et al., 2016) and Atlantic cod *Gadus morhua* L. (Forberg et al., 2011). For Dover sole, different glutaraldehyde concentrations and contact times resulted in variable hatching results and bacteria-free eggs were achieved only in a minority of the replicates, again indicating the interspecies variability. To our

knowledge, successively applying hydrogen peroxide and glutaraldehyde disinfection was not evaluated before. This combination resulted in promising results in the strive for bacteria-free Dover sole eggs. However, it was not possible to consistently obtain bacteria-free samples. This noted disparity may (partly) be attributed to a batch effect. Indeed, large differences in hatching of the control groups and therefore egg quality- were observed, highlighting an extra difficulty when developing a disinfection protocol. Although the combined glutaraldehyde and hydrogen peroxide disinfection protocol was not considered a fully-fledged step in the generation of a gnotobiotic larval model, it may be useful in small scale experiments where only a limited amount of sterile larvae are needed. Indeed, because of the benefit when housing larvae in 24-well plates, it is possible to assess the sterility status of each larva individually by analysing water samples of the well in which it is residing by means of the flow cytometer system. In this respect, it is possible to continue working with only the sterile individuals.

Ozone is not well tolerated by most of the fish species and CT values of 4 mg min L_{gas}^{-1} are most of the time the maximum tolerated ones (reviewed in De Swaef et al., 2015). The tested concentrations and contact times for Dover sole are therefore to be considered very high, and this could explain the low hatching success. Still, applying these high ozone dosages, did not result in bacteria-free eggs. In contrast, turbot (*Scophthalmus maximus*), another flatfish species known for its aquaculture potential, was found to be very resilient against ozone, tolerating CT values up to 30 mg min L_{gas}^{-1} (Grotmol et al., 2003). Finally, combining hydrogen peroxide with ozone is also not recommended for sole egg disinfection as the hatching and disinfection potential were both very low. Although the reason for the differences in tolerance between species is unknown, one hypothesis may lie herein that morphological differences between fish eggs of different species play a role (Lønning et al., 1988; Li et al., 2000; Munk & Nielsen, 2005; Fava & Toledo-Piza, 2007; Bian et al., 2010). The activity of the disinfectant may also be largely influenced by the water temperature during disinfection. Hydrogen peroxide is suggested to be increasingly toxic with rising temperature (Kiemer & Black, 1997; Small, 2004), while the activity of glutaraldehyde increases with higher temperatures (Planas & Cunha, 1999). In contrast, the activity of ozone is only affected to a limited extent by temperature (Block, 2001). It is important to keep this in mind when comparing disinfection protocols between (warm and cold water) species.

One of the aims of this study was to evaluate different disinfection protocols in order to develop a gnotobiotic model for Dover sole. Because complete elimination of the bacteria needed to be achieved, concentrations and contact times were increased to high values, compared to what is reported in the literature (De Swaef et al., 2015). This explains why hatchability decreased considerably

or was absent in many of the tested protocols. Nevertheless, some of these data may be an aid in assessing the safety and efficacy of egg disinfectants in Dover sole aquaculture.

In conclusion, a reliable experimental housing system was pinpointed for Dover sole larvae, whereby the eggs are disinfected with 1% H_2O_2 for 3 minutes and larvae are housed individually in 24-well plates. Additionally, different disinfection products and protocols were tested with variable results on hatching and bacterial load. However, none of these consistently resulted in bacteria-free larvae enabling the development of a gnotobiotic system.

5.6 ACKNOWLEDGEMENTS

Prof. dr. Paul Simoens is gratefully acknowledged for critically reviewing the manuscript. Furthermore, the authors would like to thank Lore Vandermeersch for technical support, dr. Sam Van Nevel for performing the flow cytometer analysis, Patrick Vervaet for the transportation support and IMARES Wageningen UR for providing the *S. solea* eggs. This work was supported by the Special Research Grant of Ghent University, Belgium [Bijzonder Onderzoeksfonds, grant numbers BOF12/GOA/022, BOF12/BAS/070].

*Table 5.1 - Overview of the tested disinfection protocols on Dover sole eggs, indicating the applied disinfectant products, concentrations and contact times. When two disinfection products were subsequently used, these are separated with *. The administration of antibiotics for 24 hours is also indicated. AB1=antibiotic mixture 1, consisting of 10 ppm ampicillin and 10 ppm rifampicin. AB2= antibiotic mixture 2, consisting of 10 ppm ampicillin, 10 ppm rifampicin, 150 ppm penicillin, 75 ppm streptomycin, 10 ppm oxolinic acid, 10 ppm kanamycin and 10 ppm erythromycin. For each protocol the results concerning bacterial reduction and hatching are included. Next to the number of samples without bacterial growth, also the bacterial growth on both MA and TCBS plates is expressed using a semi-quantitative score: 3 (>100 colonies/plate), 2 (10-100 colonies/plate), 1 (1-10 colonies/plate) or 0 (no bacterial growth). When a flow cytometer system was used, it is indicated whether or not bacterial growth could be found in the water samples taken at 0 or 6 DPH. The hatching rate for each incubation bottle is expressed using a semi-quantitative score (Schaeck et al., 2016): 3 ('Good', the hatchability is comparable with the eggs in the non-treated control group), 2 ('Moderate', the hatchability is good, but not as high as the non-treated control group), 1 ('low', the hatching rates is very low, compared with the non-treated control group) and 0 ('none', no hatching at all).*

Rep: replicates; #: number of samples without bacterial growth; N/A: not applicable; NB: no bacteria present; B: bacteria present

Disinfection protocol				**Sterility**												**Hatching**			
disinfectant	**concentration**	**duration (min)**	**Rep**	**MA**					**TCBS**					**Flow cytometer**					
				#	**bacterial growth**				**#**	**bacterial growth**				**0 DPH**	**6 DPH**	**hatching rate**			
					0	**1**	**2**	**3**		**0**	**1**	**2**	**3**			**0**	**1**	**2**	**3**
H_2O_2	2%	3:00	0/4	0/4			1	3	1/2	1	1			N/A	N/A		2		2
H_2O_2	4%	3:00	0/2	1/2	1	1			2/2	2				N/A	N/A			2	
H_2O_2	6%	3:00	0/2	0/2			1	1	2/2	2				N/A	N/A		2		
H_2O_2	8%	3:00	0/2	1/2	1			1	2/2	2				N/A	N/A	2			
H_2O_2	8%	5:00	0/2	0/2			1	1	2/2	2				N/A	N/A	2			
H_2O_2	10%	3:00	0/2	1/2	1		1		2/2	2				N/A	N/A	2			
H_2O_2	12%	3:00	0/2	0/2				2	2/2	2				N/A	N/A	2			
H_2O_2	3%	2:00	0/4	0/4				4	N/A					N/A	N/A		4		
H_2O_2	4%	2:00	0/2	0/2				2	N/A					N/A	N/A		2		
H_2O_2	4%	3:00	0/2	0/2				2	N/A					N/A	N/A		2		
H_2O_2	6%	2:00	0/2	0/2				2	N/A					N/A	N/A		2		
H_2O_2	8%	1:00	0/2	0/2				2	N/A					N/A	N/A		2		
H_2O_2	10%	1:00	0/2	0/2				2	N/A					N/A	N/A		2		

H_2O_2 + AB2	1%	3:00	1/4	1/4		1	3		1/2	1	1			N/A	N/A				4
H_2O_2 + AB2	2%	3:00	0/2	0/2		2			0/2		2			N/A	N/A				2
Glutaraldehyde	400 ppm	2:30	1/2	1/2	1			1	0/2			1	1	N/A	N/A				2
Glutaraldehyde	400 ppm	5:00	1/2	0/2		2			0/2		1	1		N/A	N/A	1			1
Glutaraldehyde	400 ppm	7:00	0/2	1/2	1		1		2/2	2				N/A	N/A		2		
Glutaraldehyde	400 ppm	10:00	0/2	0/2			1	1	2/2	2				N/A	N/A	2			
Glutaraldehyde + AB2	400 ppm	4:00	3/8	N/A		N/A			N/A		N/A			2x NB, 2xB	2xB	2	1		5
Glutaraldehyde + AB1	400 ppm	2:30	0/2	1/2	1	1			2/2		1	1		N/A	N/A		1		1
Glutaraldehyde + AB1	400 ppm	5:00	2/7	0/7		2	4	1	7/7	7				N/A	N/A	1	3		3
Glutaraldehyde* Glutaraldehyde	400 ppm* 400 ppm	2:30*2:30	0/2	2/2	2				2/2	2				N/A	N/A	2			
Glutaraldehyde* Glutaraldehyde + AB1	400 ppm* 400 ppm	2:30*2:30	1/2	1/2	1		1		1/2	1		1		N/A	N/A	2			
Glutaraldehyde* Glutaraldehyde + AB1	400 ppm* 400 ppm	5:00*5:00	0/3	0/3		1		2	2/3	2		1		N/A	N/A	3			
Ozone	2 mgL_{gas}^{-1}	2:00	0/2	0/2			1	1	0/2		1		1	N/A	N/A		1		1
Ozone	2 mgL_{gas}^{-1}	4:00	0/2	0/2		1	1		0/2		1	1		N/A	N/A		1	1	
Ozone	3 mgL_{gas}^{-1}	2:00	0/2	0/2		1	1		1/2	1	1			N/A	N/A	2			
Ozone	3 mgL_{gas}^{-1}	3:00	0/2	0/2		1	1		2/2	2				N/A	N/A	1	1		
Ozone	3 mgL_{gas}^{-1}	4:00	0/2	1/2	1	1			1/2	1			1	N/A	N/A		2		
Ozone	4 mgL_{gas}^{-1}	2:00	0/2	0/2		2			2/2	1				N/A	N/A	2			
Ozone	4 mgL_{gas}^{-1}	3:00	0/2	1/2	1			1	1/2	1		1		N/A	N/A	2			
Ozone + AB1	2 mgL_{gas}^{-1}	2:00	0/2	0/2				2	0/2		1	1		N/A	N/A	2			
Ozone + AB1	2 mgL_{gas}^{-1}	3:00	0/2	0/2			1	1	1/2	1		1		N/A	N/A	2			
Ozone + AB1	2 mgL_{gas}^{-1}	4:00	0/2	0/2			2		1/2	1	1			N/A	N/A	2			
H_2O_2* ozone	1%*2 mgL_{gas}^{-1}	3:00*2:00	0/2	0/2				2	0/2		2			N/A	N/A	2			
H_2O_2* ozone	1%*2 mgL_{gas}^{-1}	3:00*3:00	0/2	0/2			2		2/2	2				N/A	N/A	2			

H_2O_2* ozone	1%*2 +mgL_{gas}^{-1}	3:00*4:00	0/2	0/2				2	1/2	1		1		N/A	N/A		2	
Glutaraldehyde*ozone	400 ppm*2 mgL_{gas}^{-1}	2:00	2/2	1/2	1	1			1/2	1	1			N/A	N/A	2		
Glutaraldehyde*ozone	400 ppm*2 mgL_{gas}^{-1}	3:00	2/2	2/2	2				2/2	2				N/A	N/A	2		
Glutaraldehyde*ozone	400 ppm*2 mgL_{gas}^{-1}	4:00	2/2	1/2	1	1			1/2	1		1		N/A	N/A	2		
Glutaraldehyde*ozone + AB1	400 ppm*2 mgL_{gas}^{-1}	2:00	2/2	1/2	1			1	2/2	2				N/A	N/A	2		
Glutaraldehyde*ozone + AB1	400 ppm*2 mgL_{gas}^{-1}	3:00	2/2	0/2		2			0/2		2			N/A	N/A	2		
Glutaraldehyde*ozone + AB1	400 ppm*2 mgL_{gas}^{-1}	4:00	2/2	0/2			1	1	0/2		1	1		N/A	N/A	2		
H_2O_2*glutaraldehyde	1%*400ppm	3:00*2:30	2/4	1/4	1	3			3/4	3	1			N/A	N/A	1	1	1	1
H_2O_2*glutaraldehyde	1%*400ppm	3:00*5:00	0/2	2/2	2				2/2	2				N/A	N/A			2	
H_2O_2*glutaraldehyde + AB2	1%*400ppm	3:00*2:30	1/2	1/2	1	1			2/2	2				2xB	N/A			2	2
			2/2	N/A					N/A					B	N/A				
			0/2	N/A					N/A					B	N/A				
H_2O_2*glutaraldehyde + AB2	1%*400ppm	3:00*3:50	2/2	2/2	2				2/2	2				N/A	N/A			2	
			4/8	N/A					N/A					2xNB, 2xB	2xB		2		6
H_2O_2*glutaraldehyde + AB2	1%*400ppm	3:00*5:00	3/4	2/4	2	1	1		3/4	3	1			N/A	N/A		4		
H_2O_2*glutaraldehyde + AB2	1%*600ppm	3:00*2:00	2/2	1/2	1	1			1/2	1	1			N/A	N/A		2		
H_2O_2*glutaraldehyde + AB2	1%*800ppm	3:00*1:00	2/2	0/2			2		1/2	1	1			N/A	N/A	1		1	
H_2O_2*glutaraldehyde + AB2	2%*400 ppm	3:00*2:30	5/8	N/A					N/A					3xNB, 1xB	1xNB, 2xB		2	1	5
H_2O_2*glutaraldehyde + AB2	2%*400 ppm	3:00*3:30	2/2	N/A					N/A					NB	NB				2
			0/2	N/A					N/A					B	N/A		2		
			2/2	N/A					N/A					NB	B		2		
H_2O_2*glutaraldehyde + AB2	2%*400 ppm	3:00*4:00	4/6	N/A					N/A					2xNB, 2xB	1xNB, 1xB		1	3	2
H_2O_2*glutaraldehyde + AB2	3%*400 ppm	3:00*3:30	2/2	N/A					N/A					B	N/A			2	

H_2O_2*glutaraldehyde + AB2	3%*400 ppm	3:00*4:00	2/2	N/A			N/A		NB	N/A			1	1
H_2O_2*glutaraldehyde + AB2	4%*400 ppm	3:00*3:00	1/2	N/A			N/A		NB	B		1	1	
Glutaraldehyde*H_2O_2	400 ppm*1%	2:30*3:00	0/2	1/2	1	1	2/2	2	N/A	N/A			2	
Glutaraldehyde*H_2O_2	400 ppm*1%	7:00*3:00	1/2	1/2	1	1	2/2	2	N/A	N/A	1	1		

5.7 REFERENCES

European Commission Department Maritime Affairs and Fisheries (2016) http://ec.europa.eu/fisheries/ (accessed: 26/07/2016)

European Union DIVERSIFY project (2016) http://www.diversifyfish.eu/ (accessed: 26/07/2016)

Ahmad, F., Noldus, L.P.J.J., Tegelenbosch, R.a.J. & Richardson, M.K. (2012) Zebrafish embryos and larvae in behavioural assays. *Behaviour*, **149**, 1241-1281.

Ben-Atia, I., Lutzky, S., Barr, Y., Gamsiz, K., Shtupler, Y., Tandler, A. & Koven, W. (2007) Improved performance of gilthead sea bream, *Sparus aurata*, larvae after ozone disinfection of the eggs. *Aquaculture Research*, **38**, 166-173.

Bian, X.D., Zhang, X.M., Gao, T.X., Wan, R.J., Chen, S.Q. & Sakurai, Y. (2010) Morphology of unfertilized mature and fertilized developing marine pelagic eggs in four types of multiple spawning flounders. *Ichthyological Research*, **57**, 343-357.

Blaxter, J.H.S. (1992) The Effect of Temperature on Larval Fishes. *Netherlands Journal of Zoology*, **42**, 336-357.

Block, S.S. (2001) *Disinfection, sterilization, and preservation* /Ed. Seymour S. Block, Lippincott Williams and Wilkins.

Boglione, C., Gavaia, P., Koumoundouros, G., Gisbert, E., Moren, M., Fontagne, S. & Witten, P.E. (2013) Skeletal anomalies in reared European fish larvae and juveniles. Part 1: normal and anomalous skeletogenic processes. *Reviews in Aquaculture*, **5**, S99-S120.

Boglione, C., Gisbert, E., Gavaia, P., Witten, P.E., Moren, M., Fontagne, S. & Koumoundouros, G. (2013) Skeletal anomalies in reared European fish larvae and juveniles. Part 2: main typologies, occurrences and causative factors. *Reviews in Aquaculture*, **5**, S121-S167.

Bolle, L.J., De Jong, C.a.F., Bierman, S.M., Van Beek, P.J.G., Van Keeken, O.A., Wessels, P.W., Van Damme, C.J.G., Winter, H.V., De Haan, D. & Dekeling, R.P.A. (2012) Common Sole Larvae Survive High Levels of Pile-Driving Sound in Controlled Exposure Experiments. *Plos One*, **7**.

Braithwaite, V.A. & Huntingford, F.A. (2004) Fish and welfare: do fish have the capacity for pain perception and suffering? *Animal Welfare*, **13**, S87-S92.

Brown, N., Kling, L. & Skonberg, D. (2005) Final report to NOAA: Demonstration of Sustainable Cod Farming from Egg to Grow-out in Maine. In, p. 55. University of Maine.

Cahu, C. & Infante, J.Z. (2001) Substitution of live food by formulated diets in marine fish larvae. *Aquaculture*, **200**, 161-180.

Can, E., Saka, S. & Firat, K. (2010) Disinfection of Gilthead Sea Bream (*Sparus aurata*), Red Porgy (*Pagrus pagrus)*, and Common Dentex (*Dentex dentex*) Eggs from Sparidae with Different Disinfectants. *Kafkas Universitesi Veteriner Fakultesi Dergisi*, **16**, 299-306.

De Swaef, E., Van den Broeck, W., Dierckens, K. & Decostere, A. (2015) Disinfection of teleost eggs: a review. *Reviews in Aquaculture*, **7**, 1-21.

Devauchelle, N., Alexandre, J.C., Lecorre, N. & Letty, Y. (1987) Spawning of Sole (*Solea Solea*) in Captivity. *Aquaculture*, **66**, 125-147.

Dierckens, K., Rekecki, A., Laureau, S., Sorgeloos, P., Boon, N., Van Den Broeck, W. & Bossier, P. (2009) Development of a bacterial challenge test for gnotobiotic sea bass (*Dicentrarchus labrax*) larvae. *Environmental Microbiology*, **11**, 526-533.

Dinis, M.T., Ribeiro, L., Soares, F. & Sarasquete, C. (1999) A review on the cultivation potential of *Solea senegalensis* in Spain and in Portugal. *Aquaculture*, **176**, 27-38.

Douillet, P.A. & Holt, G.J. (1994) Surface Disinfection of Red Drum (*Sciaenops Ocellatus* Linnaeus) Eggs Leading to Bacteria-Free Larvae. *Journal of Experimental Marine Biology and Ecology*, **179**, 253-266.

Fava, D. & Toledo-Piza, M. (2007) Egg surface structure in the annual fishes Simpsonichthys (subgenera Ophthalmolebias and Xenurolebias) and Nematolebias (Teleostei : Cyprinodontiformes : Rivulidae): variability and phylogenetic significance. *Journal of Fish Biology*, **71**, 889-907.

Felip A. (Ed.), C.M.E., Herráez M.P. (Ed.), Zanuy S. (Ed.), Basurco B. (Ed.). (2009) Protocol C - Determination of egg quality and spawning performance [Pratical guide of protocols: broodstock management]. In: *Advances in fish reproduction and their application to broodstock management: a pratical manual for sea bass*, pp. 23-25, Zaragoza

Fernandez-Diaz, C., Yufera, M., Canavate, J.P., Moyano, F.J., Alarcon, F.J. & Diaz, M. (2001) Growth and physiological changes during metamorphosis of Senegal sole reared in the laboratory. *Journal of Fish Biology*, **58**, 1086-1097.

Ferraresso, S., Bonaldo, A., Parma, L., Cinotti, S., Massi, P., Bargelloni, L. & Gatta, P.P. (2013) Exploring the larval transcriptome of the common sole (*Solea solea* L.). Bmc *Genomics*, **14**.

Fonds, M. (1972) Laboratory observations on the Influence of temperature and salinity on development of the eggs and growth of the larvae of *Solea solea* (Pisces). *Marine Ecology Progress Series*, **1**, 91-99.

Forberg, T., Arukwe, A. & Vadstein, O. (2011) A protocol and cultivation system for gnotobiotic Atlantic cod larvae (*Gadus morhua* L.) as a tool to study host microbe interactions. *Aquaculture*, **315**, 222-227.

Fukuhara, O. (1990) Effects of Temperature on Yolk Utilization, Initial Growth, and Behavior of Unfed Marine Fish-Larvae. *Marine Biology*, **106**, 169-174.

Gatesoupe, F.J., Lambert, C. & Nicolas, J.L. (1999) Pathogenicity of *Vibrio splendidus* strains associated with turbot larvae, *Scophthalmus maximus*. *Journal of Applied Microbiology*, **87**, 757-763.

Goncalves, A.A. & Gagnon, G.A. (2011) Ozone Application in Recirculating Aquaculture System: An Overview. *Ozone-Science & Engineering*, **33**, 345-367.

Gosselin, S., Fortier, L. & Gagne, J.A. (1989) Vulnerability of Marine Fish Larvae to the Toxic Dinoflagellate Protogonyaulax Tamarensis. *Marine Ecology Progress Series*, **57**, 1-10.

Grotmol, S., Dahl-Paulsen, E. & Totland, G.K. (2003) Hatchability of eggs from Atlantic cod, turbot and Atlantic halibut after disinfection with ozonated seawater. *Aquaculture*, **221**, 245-254.

Imsland, A.K., Foss, A., Conceicao, L.E.C., Dinis, M.T., Delbare, D., Schram, E., Kamstra, A., Rema, P. & White, P. (2003) A review of the culture potential of *Solea solea* and *S. senegalensis*. *Reviews in Fish Biology and Fisheries*, **13**, 379-407.

Interaminense, J.A., Calazans, N.F., Do Valle, B.C., Vogeley, J.L., Peixoto, S., Soares, R. & Lima, J.V. (2014) *Vibrio* spp. Control at Brine Shrimp, *Artemia*, Hatching and Enrichment. *Journal of the World Aquaculture Society*, **45**, 65-74.

Ishimaru, K., Akagawamatsushita, M. & Muroga, K. (1996) *Vibrio ichthyoenteri* sp nov, a pathogen of Japanese flounder (*Paralichthys olivaceus*) larvae. *International Journal of Systematic Bacteriology*, **46**, 155-159.

Kiemer, M.C.B. & Black, K.D. (1997) The effects of hydrogen peroxide on the gill tissues of Atlantic salmon, *Salmo salar* L. *Aquaculture*, **153**, 181-189.

Kohn, D.F., Martin, T.E., Foley, P.L., Morris, T.H., Swindle, M.M., Vogler, G.A. & Wixson, S.K. (2007) Guidelines for the assessment and management of pain in rodents and rabbits. *Journal of the American Association for Laboratory Animal Science*, **46**, 97-108.

Lane, A., Hough, C. & Bostock, J. (2014) The long-term economic and ecologic impact of larger sustainable aquaculture. Study for the European Parliament, Directorate General for Internal Policies, Policy Department B, Structural and Cohesion Policies—Fisheries.

Li, X., Defoirdt, T., Yang, Q., Laureau, S., Bossier, P. & Dierckens, K. (2014) Host-induced increase in larval sea bass mortality in a gnotobiotic challenge test with *Vibrio anguillarum*. *Diseases of Aquatic Organisms*, **108**, 211-216.

Li, Y.W., C. & Yang, J. (2000) Comparative ultrastructural studies of the zona radiata of marine fish eggs in three genera in Perciformes. *Journal of Fish Biology*, **56**, 615-621.

Lønning, S., Kjorsvik, E. & Falkpetersen, I.B. (1988) A Comparative-Study of Pelagic and Demersal Eggs from Common Marine Fishes in Northern Norway. *Sarsia*, **73**, 49-60.

Makridis, P., Bergh, Ø., Skjermo, J. & Vadstein, O. (2001) Addition of bacteria bioencapsulated in *Artemia* metanauplii to a rearing system for halibut larvae. *Aquaculture International*, **9**, 225-235.

Makridis, P., Jon Fjellheim, A., Skjermo, J. & Vadstein, O. (2000) Colonization of the gut in first feeding turbot by bacterial strains added to the water or bioencapsulated in rotifers. *Aquaculture International*, **8**, 367-380.

Martin, I., Rasines, I., Gomez, M., Rodriguez, C., Martinez, P. & Chereguini, O. (2014) Evolution of egg production and parental contribution in Senegalese sole, *Solea senegalensis*, during four consecutive spawning seasons. *Aquaculture*, **424**, 45-52.

Morehead, D.T. & Hart, P.R. (2003) Disinfection of striped trumpeter (*Latris lineata*) eggs with glutaraldehyde. *Aquaculture International*, **11**, 255-260.

Munk P. & Nielsen J. (2005) *Eggs and larvae of North Sea fishes,* biofolia, Denmark.

Munro, P.D., Barbour, A. & Birkbeck, T.H. (1995) Comparison of the Growth and Survival of Larval Turbot in the Absence of Culturable Bacteria with Those in the Presence of *Vibrio Anguillarum, Vibrio Alginolyticus,* or a Marine *Aeromonas* Sp. *Applied and Environmental Microbiology*, **61**, 4425-4428.

Norton, W.H.J. (2013) Toward developmental models of psychiatric disorders in zebrafish. *Frontiers in Neural Circuits*, **7**.

OECD (2013) Test No. 236: Fish embryo acute toxicity (FET) test, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.

Palazzi, R., Richard, J., Bozzato, G. & Zanella, L. (2006) Larval and juvenile rearing of common sole (*Solea solea* L.) in the Northern Adriatic (Italy). *Aquaculture*, **255**, 495-506.

Panini, E.B., Mylonas, C.C., Zanuy, S., Carrillo, M., Ramos, J. & Bruce, M.P. (2001) Incubation of embryos and larvae of marine fish using microtiter plates. *Aquaculture International*, **9,** 189-195.

Parma, L., Bonaldo, A., Massi, P., Yufera, M., Martinez-Rodriguez, G. & Gatta, P.P. (2013) Different early weaning protocols in common sole (*Solea solea* L.) larvae: Implications on the performances and molecular ontogeny of digestive enzyme precursors. *Aquaculture*, **414**, 26-35.

Pepin, P., Orr, D.C. & Anderson, J.T. (1997) Time to hatch and larval size in relation to temperature and egg size in Atlantic cod (*Gadus morhua*). *Canadian Journal of Fisheries and Aquatic Sciences*, **54**, 2-10.

Pfeffer, C. & Oliver, J.D. (2003) A comparison of thiosulphate-citrate-bile salts-sucrose (TCBS) agar and thiosulphate-chloride-iodide (TCI) agar for the isolation of *Vibrio* species from estuarine environments. *Letters in Applied Microbiology*, **36**, 150-151.

Planas, M. & Cunha, I. (1999) Larviculture of marine fish: problems and perspectives. *Aquaculture*, **177**, 171-190.

Planas, M., Perez-Lorenzo, M., Hjelm, M., Gram, L., Fiksdal, I.U., Bergh, O. & Pintado, J. (2006) Probiotic effect *in vivo* of *Roseobacter* strain 27-4 against *Vibrio (Listonella) anguillarum* infections in turbot (*Scophthalmus maximus* L.) larvae. *Aquaculture*, **255**, 323-333.

Rawls, J.F., Samuel, B.S. & Gordon, J.I. (2004) Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 4596-4601.

Reid, H.I., Treasurer, J.W., Adam, B. & Birkbeck, T.H. (2009) Analysis of bacterial populations in the gut of developing cod larvae and identification of *Vibrio logei, Vibrio anguillarum* and *Vibrio splendidus* as pathogens of cod larvae. *Aquaculture*, **288**, 36-43.

Richard, P., Bergeron, J.P., Boulhic, M., Galois, R. & Personleruyet, J. (1991) Effect of Starvation on RNA, DNA and Protein-Content of Laboratory-Reared Larvae and Juveniles of *Solea Solea*. *Marine Ecology Progress Series*, **72**, 69-77.

Russell, W.M.S. & Burch, R.L. (1959) *The Principles of Humane Experimental Technique*. Methuen, London.

Salvesen, I., Oie, G. & Vadstein, O. (1997) Surface disinfection of Atlantic halibut and turbot eggs with glutaraldehyde: Evaluation of concentrations and contact times. *Aquaculture International*, **5**, 249-258.

Samson, J.C., Shumway, S.E. & Weis, J.S. (2008) Effects of the toxic dinoflagellate, *Alexandrium fundyense* on three species of larval fish: a food-chain approach. *Journal of Fish Biology*, **72**, 168-188.

Sandlund, N., Rodseth, O.M., Knappskog, D.H., Fiksdal, I.U. & Bergh, O. (2010) Comparative susceptibility of turbot, halibut, and cod yolk-sac larvae to challenge with *Vibrio* spp. *Diseases of Aquatic Organisms*, **89**, 29-37.

Schaeck, M., De Swaef, E., Van Den Broeck, W., Van Nevel, S., Boon, N., De Geyter, N., Morent, R., Demeestere, K., Duchateau, L., Coulombet, C., Haesebrouck, F. & Decostere, A. (2016) Germ-free sea bass *Dicentrarchus labrax* larval model: a valuable tool in the study of host-microbe interactions. *Diseases of Aquatic Organisms*, **117**, 177-185.

Shikongo-Nambabi, M.N.N.N., Kachigunda, B. & Venter, S.N. (2010) Evaluation of oxidising disinfectants to control *Vibrio* biofilms in treated seawater used for fish processing. *Water SA*, **36**, 215-220.

Situmorang, M.L., Dierckens, K., Mlingi, F.T., Van Delsen, B. & Bossier, P. (2014) Development of a bacterial challenge test for gnotobiotic Nile tilapia *Oreochromis niloticus* larvae. *Diseases of Aquatic Organisms*, **109**, 23-33.

Skjermo, J. & Bergh, O. (2004) High-M alginate immunostimulation of Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae using *Artemia* for delivery, increases resistance against vibriosis. *Aquaculture*, **238**, 107-113.

Small, B.C. (2004) Accounting for water temperature during hydrogen peroxide treatment of channel catfish eggs. *North American Journal of Aquaculture*, **66**, 162-164.

Sneddon, L.U. (2003) The evidence for pain in fish: the use of morphine as an analgesic. *Applied Animal Behaviour Science*, **83**, 153-162.

Sneddon, L.U. (2009) Pain Perception in Fish: Indicators and Endpoints. *Ilar Journal*, **50**, 338-342.

Sneddon, L.U. (2015) Pain in aquatic animals. *Journal of Experimental Biology*, **218**, 967-976.

Sorgeloos, P., Food, Nations, A.O.O.T.U. & Landbouwwetenschappen, R.T.G.F.V.D. (1986) Manual for the culture and use of brine shrimp artemia in aquaculture, State University of Ghent, Faculty of Agriculture.

Thompson, F.L., Iida, T. & Swings, J. (2004) Biodiversity of vibrios. *Microbiology and Molecular Biology Reviews*, **68**, 403-+.

Thomson, R., Macpherson, H.L., Riaza, A. & Birkbeck, T.H. (2005) *Vibrio splendidus* biotype 1 as a cause of mortalities in hatchery-reared larval turbot, *Scophthalmus maximus* (L.). *Journal of Applied Microbiology*, **99**, 243-250.

Trang, T.T. (2013) Identification of bacteria from the aquaculture food chain isolated on thiosulphate citrate bile salt medium (TCBS). Master thesis, Master of Science in Aquaculture, Faculty of Biosciences Engineering, University of Ghent, Belgium, 57pp.

Verner-Jeffreys, D.W., Nakamura, I. & Shields, R.J. (2007) Surface disinfection of Pacific threadfin, *Polydactylus sexfilis*, and amberjack, *Seriola rivoliana*, eggs. *Aquaculture Research*, **38**, 605-612.

Villamil, L., Figueras, A., Toranzo, A.E., Planas, M. & Novoa, B. (2003) Isolation of a highly pathogenic *Vibrio pelagius* strain associated with mass mortalities of turbot, *Scophthalmus maximus* (L.), larvae. *Journal of Fish Diseases*, **26**, 293-303.

Wang, W., Wang, H., Yu, C.Y. & Jiang, Z.Q. (2015) Acute toxicity of ammonia and nitrite to different ages of Pacific cod (*Gadus macrocephalus*) larvae. *Chemical Speciation and Bioavailability*, 27, 147-155.

West, P.A., Russek, E., Brayton, P.R. & Colwell, R.R. (1982) Statistical Evaluation of a Quality-Control Method for Isolation of Pathogenic *Vibrio* Species on Selected Thiosulfate-Citrate Bile-Salts Sucrose Agars. *Journal of Clinical Microbiology*, **16**, 1110-1116.

Williams, T.C., Froelich, B. & Oliver, J.D. (2013) A new culture-based method for the improved identification of *Vibrio vulnificus* from environmental samples, reducing the need for molecular confirmation. *Journal of Microbiological Methods*, **93**, 277-283.

Chapter 6 EXPERIMENTAL INFECTION MODEL FOR VIBRIOSIS IN DOVER SOLE *SOLEA SOLEA* LARVAE AS AN AID IN STUDYING ITS PATHOGENESIS AND ALTERNATIVE TREATMENTS

Based on: De Swaef, E., Vercauteren, M., Duchateau, L., Haesebrouck, F., Decostere, A. (2018) Experimental infection model for vibriosis in Dover sole (*Solea solea*) larvae as an aid in studying its pathogenesis and alternative treamtents. *Veterinary Research*, **49**, 24.

6.1 ABSTRACT

Severe economic losses due to diseases in marine larviculture may be linked to vibriosis. To better understand the pathogenesis of vibriosis and evaluate new ways to prevent and combat this important disease, there is a great need for reliable and reproducible experimental infection models. The present study aimed at developing a challenge model for vibriosis in Dover sole larvae and testing its applicability to study the effect of the probiotic treatment. For that purpose, larvae were challenged at 10 days post hatching with *Vibrio anguillarum* WT, *V. anguillarum* HI610 or *V. harveyi* WT. Following administration of *V. anguillarum* WT via immersion at 1×10^7 colony forming units/mL, a larval mortality of 50% was observed at 7 days post-inoculation (17 days after hatching). In a next step, the probiotic potential of 371 isolates retrieved from Dover sole was assessed by screening for their inhibitory effects against *Vibrio* spp. and absence of haemolytic activity. One remaining isolate (*V. proteolyticus*) and *V. lentus*, known for its protective characteristics in seabass larvae, were further tested in vivo by means of the pinpointed experimental infection model. Neither isolate provided via the water or feed proved to be protective for the Dover sole larvae against challenge with *V. anguillarum* WT. This developed challenge model constitutes a firm basis to expedite basic and applied research regarding the pathogenesis and treatment of vibriosis as well as for studying the impact of (a)biotic components on larval health.

6.2 INTRODUCTION

Dover sole (*Solea solea*) is greatly appreciated in high quality restaurants and has a high market value, making it a very promising candidate for European aquaculture (Rodgers et al., 2005; Bjørndal et al., 2016). In addition, farmers developed a renewed interest in Dover sole aquaculture to diversify their operations due to indications of limited market growth for species such as sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) (Bjørndal et al., 2016; FAO, 2016). Furthermore, a reliable sole production would reduce fishing pressure on wild Dover sole populations, whereby the main sole stocks only recently recovered after collapsing 20 years ago and are now at or close to being harvested sustainable (Bjørndal et al., 2016; FAO, 2016). As for other marine teleost species, high larval mortality rates (especially during first feeding) and limited knowledge on the nutritional requirements result in juvenile scarcity for stocking purposes, being the main obstacle for large scale aquaculture (Makridis et al., 2008; Ferraresso et al., 2013; Bjørndal et al., 2016).

One of the major causes for the low and unpredictable survival in marine larviculture are outbreaks of infectious diseases. Vibriosis is one of the most challenging bacterial diseases to tackle in these early life stages (Thompson et al., 2004; Austin & Austin, 2012; Novriadi, 2016) and multiple publications stress the importance of pathogenic *Vibrio* species in hatcheries and their potential to cause disease (Touraki et al., 2012; D'Alvise et al., 2013; Silva et al., 2014). The causative agents of vibriosis are bacteria belonging to the genus *Vibrio*, with *Vibrio anguillarum* being the most prominent member (Toranzo et al., 2015; Novriadi, 2016). Important contributions are made to prevent and control infectious diseases, in the past mainly focussing on the use of antimicrobial agents or chemical additives (Cabello, 2006). However, the emerging antimicrobial resistance, the potential transfer of antimicrobial resistance genes to fish or human pathogens (Romero et al., 2012) and the possibility that antimicrobials can enter the human food chain (Willis, 2000), stress the need to develop reliable alternatives. These latter should ensure a healthy microbial environment in the larval rearing tanks and hence decrease disease and mortality (Diaz-Rosales et al., 2009). Various environmentally-friendly prophylactic disease treatments are currently being pinpointed for marine larvae including probiotics (Verschuere et al., 2000; Nayak, 2010; De et al., 2014), prebiotics (Bricknell & Dalmo, 2005; Ringø et al., 2010) and immunostimulants (Ringø et al., 2012), with hitherto no data are available on the use of such treatments including probiotics in Dover sole larvae. In addition, there is a clear paucity of information in our understanding of the mode of action of probiotics and their interaction with the aquatic organism especially in the marine larval stage (Vine et al., 2006; Tinh et al., 2008; Ringø et al., 2010). To remediate this and to elucidate the mechanism by which these treatments exert their beneficial impact, more knowledge on how the bacterium interacts with its host and causes disease is needed. For that purpose, the availability of reliable experimental infection models is imperative. Only

a limited number of studies succeeded in developing such models for marine fish larvae. Significant mortality was noted following challenge of turbot larvae (*Scophthalmus maximus*) with *V. anguillarum* HI610 (Planas et al., 2005) and sea bass larvae with *V. anguillarum* HI610 (Dierckens et al., 2009) or *V. harveyi* (Schaeck et al., 2016a). For Dover sole, an experimental multi well plate housing system was pinpointed (De Swaef et al., 2017). However, a reproducible and reliable experimental infection model eliciting vibriosis is non-existing, hampering in-depth research on the interplay between *Vibrio* and its larval host.

In this respect, the present study aimed at developing the first experimental infection model for vibriosis in Dover sole larvae. In addition, the protective potential of probiotic candidates for Dover sole was evaluated in vitro and subsequently in vivo by means of the pinpointed challenge model.

6.3 MATERIAL AND METHODS

All experiments were approved by the Ethical Committee of the Faculty of Veterinary Medicine and Bio-engineering Sciences, Ghent University (no. EC2015/28, EC2015/70 and EC2015/73).

6.3.1 *Solea solea* larvae

Solea solea eggs were obtained from the Wageningen Marine Research (Ijmuiden, the Netherlands) and Stichting Zeeschelp (Kamperland, the Netherlands). Eggs were naturally spawned overnight and collected the next morning. The dead eggs were removed, whereafter transportation to the research facilities in natural seawater (32 g/L) occurred. Upon arrival, eggs were acclimatized with artificial seawater (ASW) of 34 g/L (Instant Ocean, Aquarium Systems, Mentor, Ohio) and further incubated herein under aeration. One day after arrival, dead eggs were removed and developing Dover sole eggs were disinfected with 1% H_2O_2 for 3 min (De Swaef et al., 2017). After disinfection, eggs were kept in 400 mL aerated autoclaved artificial seawater (AASW, Instant Ocean) in glass bottles at 16 ± 1 °C, each bottle containing approximately 600 embryos. Housing the larvae was performed as described by (De Swaef et al., 2017). Two days after hatching (DAH), larvae were placed individually in 24-well plates, incubated at 16 ± 1 °C and fed ad libitum with sterile *Artemia franciscana* nauplii (EG type; INVE Aquaculture NV, Belgium) every other day, starting from 6 DAH onwards, except when indicated otherwise. Sterile *Artemia* cysts and nauplii were obtained through decapsulation (Sorgeloos et al., 1986). Half of the well water was replaced every other day and all larvae were subjected to a circadian rhythm of 9 h light and 15 h darkness.

6.3.2 Bacterial isolates

6.3.2.1 Experimental infection model

Three *Vibrio* strains were adopted. *Vibrio anguillarum* HI610 was originally isolated from diseased Atlantic cod (*Gadus morhua*) (Samuelsen et al., 2003). *Vibrio anguillarum* WT and *V. harveyi* WT strains were both procured from a disease outbreak in a French sea bass farm and subjected to minimal *in vitro* passaging.

6.3.2.2 In vitro *selection of probiotic candidates*

A total of 371 isolates retrieved from Dover sole larvae or the intestine of adults (both wild caught individuals and animals that were housed for 2-3 months in experimental facilities) were screened for their antagonism against *V. anguillarum* HI610, *V. anguillarum* WT or *V. harveyi* WT using the Kirby-Bauer disk diffusion method (NCCLS, 1997) as described in (Schaeck et al., 2016b). Briefly, the presence or absence of an inhibition zone surrounding disks immersed in the cultivated broth of the probiotic candidates following incubation, was recorded. The isolates eliciting growth inhibition were tested for their haemolytic activity by inoculating Marine Agar (MA, Scharlab S.L., Sentmenat, Spain) plates supplemented with 5% sheep blood (Oxoid Ltd, Hampshire, UK) with the cultivated broth of the probiotic candidates. Haemolytic activity was examined after 48 h incubation at 18 °C. Probiotic candidates exhibiting inhibition against at least one of the tested *Vibrio* strains and without haemolytic activity were identified by means of 16S rRNA gene sequencing. Therefore, the genomic DNA was extracted according to (Declercq et al., 2013) and the 16S rRNA gene was amplified (Smet et al., 2012). In short, amplification of the 16S rRNA gene was performed using the commercially available Qiagen Taq Mastermix and primers αβ-NOT (5'-TCAAACTAGGACCGAGTC-3') and ωMB (5'-TACCTTGTTACTTCACCCCA-3') as described by Baele et al. (2001). PCR products were sequenced using the BigDye Terminator sequencing kit (Applied Biosystems) and primers pD, Gamma*, 3 and O* (Coenye et al., 1999). Sequences were determined on an automatic DNA sequencer (ABI Prism 3100 Genetic analyser; Applied Biosystems) and identified using the program BLAST and the NCBI/GenBank. Species known to be potentially zoonotic were excluded from further experiments.

Vibrio lentus isolated from clinically healthy seabass larvae (10 DAH) and proven to significantly reduce mortality of seabass larvae after challenge with *V. harveyi* WT (Schaeck et al., 2016b), was also included as a probiotic candidate. *Vibrio lentus* showed in vitro inhibition against *V. anguillarum* HI610 and *V. harveyi* WT and proved to be non-haemolytic (Schaeck et al., 2016b). The inhibitory effect against *V. anguillarum* WT was tested as described above.

6.3.3 *In vivo* experiments

For each experimental trial, Dover sole eggs from one single batch were used and a negative control group was included in which larvae underwent the same physical handling and water exchanges but without the addition of bacterial cells. Each group consisted of 96 larvae at 4 DAH, divided over four 24-well plates filled with AASW. At the end of each experiment all remaining larvae were sacrificed by immersion in an overdose of MS 222 (tricaine methanesulfonate, Sigma-Aldrich, Diegem, Belgium).

6.3.3.1 Bacterial cultivation practices

All bacterial isolates were grown for 48 h at 18 °C on MA, followed by cultivation in tryptic soy broth (TSB, Becton, Dickinson and Company, New Jersey, USA) supplemented with 1.5% NaCl for 24 h at 18 °C. Cells were harvested by centrifugation at 3500 rpm for 10 min. The resulting pellet was washed twice with AASW and subsequently resuspended in AASW. Optical densities were determined using an ATB 1550 densitometer (BioMérieux, Marcy-l'Etoile, France). Bacterial titres were verified by making a tenfold dilution series in triplicate on MA plates, prior to administration.

6.3.3.2 Development of the experimental infection model

In the first challenge experiment, three groups were challenged with either *V. anguillarum* HI610, *V. anguillarum* WT or *V. harveyi* WT. The *Vibrio* strains were added to the well water of 10 DAH larvae at a final concentration of 1×10^5 colony forming units (CFU)/mL. In the second experiment, the same groups were included but the *Vibrio* strains were added so as to achieve a final concentration of 1×10^6 CFU/mL. In the third experiment, only one group was challenged with *V. anguillarum* WT resulting in a final concentration of 1×10^7 CFU/mL.

Six hours following the inoculation with the *Vibrio* strains, half of the well water was replaced. From the next day onwards, the normal feeding regime with sterile *Artemia* nauplii and water replacement every other day were started. Larval mortality was monitored daily up to 7 days post inoculation (DPI) (17 DAH).

6.3.3.3 Assessment of the protective potential of probiotic candidates

6.3.3.3.1 Harmfulness of the probiotic candidates to Dover sole larvae

The harmfulness of the resulting probiotic candidate 1 and of *V. lentus* was tested in two separate experiments. Bacterial cells were added to the well water of larvae at 4, 6 and 8 DAH resulting in a final concentration of 1×10^7 CFU/mL. Larval mortality was monitored daily up to 17 DAH. The standard body length of all remaining larvae was measured using an Olympus SZX7 stereomicroscope and cell D software (Soft imaging system, Olympus NV).

6.3.3.3.2 Protection of Dover sole larvae against challenge with *V. anguillarum* WT

In the first experiment, the larvae of two experimental groups were provided with probiotic candidate 1 or *V. lentus* via the well water on 4, 6 and 8 DAH in a final concentration of 1×10^7 CFU/mL. Subsequently, the larvae were challenged with *V. anguillarum* WT at a final concentration of 1×10^7 CFU/mL at 10 DAH. A third group (positive control) was inoculated with *V. anguillarum* WT without being previously administered a probiotic candidate.

In the second experiment the same experimental groups were included but probiotic candidates were supplied via the feed. Therefore, *Artemia* cysts were incubated at a concentration of 1.2 g/L at 28°C for 24h. Next, newly hatched sterile *Artemia* nauplii were incubated at 20 °C for 6 h in a suspension (1×10^7 CFU/mL) of one of the two probiotic candidates. Subsequently, the *Artemia* nauplii were washed and fed to the Dover sole larvae at 5 DAH and 7 DAH. To evaluate the bacterial concentration present on the surface and inside of the *Artemia* nauplii, a subsample of at least 20 rinsed *Artemia* nauplii were homogenised and resuspended in 100 µL AASW. Bacterial titres were verified by making a tenfold dilution series of the homogenate on MA. From 9 DAH onwards, the normal feeding regime with sterile *Artemia* nauplii every other day was started.

In both experiments, mortality was monitored daily up to 21 DAH.

6.3.4 Statistical analysis

For the experimental infection model, the survival (0-1) at the end of the study was compared between the three *Vibrio* strains (in different concentrations) and the negative control group using a logistic regression model. In the harmfulness study, the survival (0-1) of the negative control was compared with the probiotic candidates by a logistic regression model. The body length measurements of the larvae exposed to the probiotic candidates in comparison with the negative control group were analyzed within each experiment by a linear fixed effects model. To evaluate the protective potential of the probiotic candidates, administered via the water or the food, the survival (0-1) at the end of the study was compared between the negative control, the positive control and probiotic candidates by a logistic regression model.

All analyses were performed using SAS version 6.4. The global significance level of 5% was used but multiple comparisons significance levels were adjusted based on the Bonferroni correction method in order to compare the outcome of the challenges with the three *Vibrio* isolates with the negative control and to compare the probiotic candidate treatments, negative control and the positive control group (comparison wise significance level set at 0.05/3=0.0167).

6.4 RESULTS

6.4.1 Experimental infection model

In the first challenge experiment to develop the infection model, no significant difference in survival at 7 DPI (17 DAH) was observed between the negative control group (survival of 91%) and the larvae inoculated with 10^5 CFU/mL *V. anguillarum* HI610 (survival of 86%) (OR = 0.66, 95% CI:[0.26;1.66], $p = 0.367$), *V. anguillarum* WT (survival of 84%) (OR = 0.56, 95% CI:[0.23;1.37], $p = 0.195$) and *V. harveyi* (survival of 94%) (OR = 1.55, 95% CI:[0.52;4.64], $p = 0.423$). In the second challenge experiment, no significant difference in survival at 7 DPI (17 DAH) was noted between the negative control group (survival of 90%) and the larvae inoculated with 10^6 CFU/mL *V. anguillarum* HI610 (survival of 93%) (OR = 1.48, 95% CI:[0.53;4.16], $p = 0.448$) or *V. harveyi* WT (survival of 86%) (OR = 0.90, 95% CI:[0.36;2.27], $p = 0.817$). Following challenge with *V. anguillarum* WT at 10^6 CFU/mL, a statistically significant reduction in survival to 61% (OR = 0.19, 95% CI:[0.08;0.41], $p < 0.001$) was discerned. In the third experiment, a significantly reduced survival of 50% ($p < 0.001$) was detected between the larvae inoculated with *V. anguillarum* WT at 10^7 CFU/mL and the negative control group (survival of 92%) [OR = 0.09, 95% CI:[0.04;0.21], $p < 0.001$) (Table 6.1).

6.4.2 Assessment of the protective potential of probiotic candidates

6.4.2.1 In vitro *selection of probiotic candidates*

None of the isolates retrieved from adult Dover sole displayed in vitro inhibition against one of the tested *Vibrio* strains. Four probiotic candidates recovered from Dover sole larvae were selected based on the presence of in vitro inhibition against the three tested *Vibrio* strains and the absence of haemolytic activity. Following 16S rRNA sequencing, for three probiotic candidates the indicative species name was *V. parahaemolyticus* with 99% sequence homology and hence these isolates were excluded from further assays due to their potential zoonotic character. The remaining probiotic candidate was indicatively identified as *V. proteolyticus* with 99% sequence homology and further analysed in vivo for its protective potential.

Vibrio lentus isolated from seabass larvae demonstrated in vitro inhibition against *V. anguillarum* WT.

6.4.2.2 Harmfulness of the probiotic candidates to Dover sole larvae

In the first experiment, no significant difference in survival at 17 DAH was observed between the negative control group (survival of 76%) and the larvae inoculated with *V. proteolyticus* (survival of 74%) (OR = 0.89 [95% CI:0.46;1.74], $p = 0.739$). Also in the second experiment, no significant difference in survival at 17 DAH was observed between the negative control group (survival of 89%) and the larvae inoculated with *V. lentus* (survival of 88%) (OR = 0.89 [95% CI:0.46;1.74], $p = 0.824$). In both experiments the estimated odds ratios were close to one. No significant difference in standard body

length was observed at 17 DAH between the control group and the group inoculated with *V. proteolyticus* (p = 0.3223). By 17 DAH, the larvae inoculated with *V. lentus* had a mean standard body length of 5570.49 µm (SD = 749.13 µm), which is significantly higher than what was noted in the larvae of the control group, 5000.06 µm (SD = 421.73 µm) (p = 0.006) (Table 6.2).

6.4.2.3 Protection of Dover sole larvae against challenge with V. anguillarum *WT*

In the first experiment, no significant differences in survival at 21 DAH were observed between the positive control group (survival of 35%) and the larvae inoculated before challenge via the water with *V. proteolyticus* (survival of 37%) (OR = 1.05 [95% CI:0.57;1.91], p = 0.880) or with *V. lentus* (survival of 34%) (OR = 0.96, 95% CI:[0.52;1.75], p = 0.880). A significant difference in survival at 21 DAH was discerned between the negative control group (survival of 80%) and the larvae inoculated with *V. proteolyticus* (OR = 0.14 [95% CI:0.07;0.26], p < 0.001) or with *V. lentus* (OR = 0.14 [95% CI:0.07;0.26], p < 0.001) prior to challenge (Table 6.3).

In the second experiment, no significant differences in survival at 21 DAH were noted between the positive control group (survival of 52%) and the larvae that were inoculated via the feed before being challenged with *V. proteolyticus* (survival of 45%) (OR = 0.75 [95% CI:0.42;1.33], p = 0.312) or with *V. lentus* (survival of 49%) (OR = 0.88, 95% CI:[0.50;1.57], p = 0.665). A significant difference in survival at 21 DAH was noticed between the negative control group (survival of 72%) and the larvae inoculated with *V. proteolyticus* (OR = 0.32 [95% CI:0.17;0.59], p < 0.001) or with *V. lentus* (OR = 0.38 [95% CI:0.20;0.69], p = 0.001) before challenge (Table 6.3). The bacterial concentration of the *Artemia* nauplii ranged between 6.3 x10^4 and 1.9 x 10^5 CFU/*Artemia* for *V. proteolyticus* and between 1.3 x 10^5 and 4.4 x 10^5CFU/*Artemia* for *V. lentus*.

6.5 DISCUSSION

Infectious diseases (e.g. vibriosis) are a major cause of marine larval mortality and various environmentally-friendly prophylactic treatments are currently being pinpointed including the use of pro- and prebiotics. However, very limited data on these alternative treatments are available for Dover sole. In the present study, an experimental challenge model using *V. anguillarum* WT was developed to reproduce vibriosis in Dover sole and therefore provide a building block to move forward in the assessment of novel therapies and the elucidation of the pathogenesis of vibriosis. This model was then used for evaluating the protective potential of two probiotic candidates, selected using in vitro criteria.

Only a limited number of studies focus on the development of challenge models in marine fish larvae. These may be performed by bioencapsulation of the pathogen in live feed (Muroga et al., 1990; Munro

et al., 1995; Planas et al., 2005) or challenge via the water. When pathogen delivery is performed via the water, the challenge experiments are mostly conducted in multiwell plate systems (Bergh et al., 1997; Sandlund et al., 2008; 2010; Schaeck et al., 2016a). In a minority of studies, larvae are housed in small groups in vials (Dierckens et al., 2009; Li et al., 2014). However, in these vials an increase virulence of *V. anguillarum* depending on the number of dead larvae was observed (Li et al., 2014). This indicates that (remnants of) death larvae can have an impact on living animals or the pathogen, increasing variability between replicates. The challenge model as proposed in the current study draws on a multi well housing system that was developed previously (De Swaef et al., 2017). Housing the larvae individually offers the advantage that the possible death of one larva has no effect on the other larvae, rendering these experiments more reproducible. Furthermore, the health status and behaviour of individually housed larvae may be monitored more easily. In the present study, the pathogenicity of three bacterial strains was evaluated and the strain and concentration eliciting around 50% mortality within 6 days following challenge selected. Indeed, the induced mortality needs to be sufficiently high to enable the investigation of the protective effect of prophylactic or curative treatments. On the other hand, a too severe challenge model is not appropriate neither as this would hinder detection of a possible protective capacity.

Vibriosis is reported as a cause of disease/mortality in as many as at least 48 species of marine fish (Austin & Austin, 2007). The importance of pathogenic *Vibrio* species in hatcheries and their potential to cause disease is stressed by various authors (Touraki et al., 2012; D'Alvise et al., 2013; Silva et al., 2014). *Vibrio anguillarum* is described as the causative agent of vibriosis in the young life stages of at least 12 fish species. The number of disease case reports in Dover sole culture is limited and involves adults (Manfrin et al., 2003) and juveniles (Paolini et al., 2010). This scarce information on Dover sole health management is largely rooted in the fact that Dover sole aquaculture was initiated only relatively recently, underscoring the need for research on health and disease in this alternative aquaculture species. A *V. anguillarum* isolate not originally retrieved from sole was included that induced larval mortality in other marine species. Indeed, although fish species-specific virulence was described for different *Vibrio* species, several strains originating from a disease outbreak in (larvae of) one fish species were able to cause mortality in another (Sandlund et al., 2010; Frans et al., 2013; Rønneseth et al., 2017). To exemplify this, although *V. anguillarum* strain 87-9-117 was originally isolated from rainbow trout (*Oncorhynchus mykiss* Walbaum), it caused high mortality in sea bass larvae, indicating that there is no stringent host-specificity for vibriosis (Rønneseth et al., 2017). In the present study, normal survival was obtained for Dover sole larvae subsequent to inoculation with *V. harveyi* WT retrieved from diseased sea bass and pathogenic to sea bass larvae (Schaeck et al., 2016a). Indeed, in a previous study challenge with *V. harveyi* resulted in 70% mortality in sea bass larvae

following administration at 1×10^5 CFU/mL (Schaeck et al., 2016b). The latter is also a well-known causative agent of vibriosis in adults of the closely related Senegalese sole (*Solea senegalensis* Kaup) (Arijo et al., 2005; Seljestokken et al., 2006). Secondly, no increased mortality was observed following challenge of the Dover sole larvae with *V. anguillarum* HI610 at concentrations up to 1×10^6 CFU/mL. This bacterial strain originating from diseased Atlantic cod larvae (Samuelsen et al., 2003), elicited high mortality rates in several experimental challenge tests including yolk sac larvae of turbot (Sandlund et al., 2010; Rønneseth et al., 2017), halibut (*Hippoglossus hippoglossus*) (Sandlund et al., 2010; Rønneseth et al., 2017), Atlantic cod (Seljestokken et al., 2006; Sandlund et al., 2010) and seabass (Dierckens et al., 2009; Schaeck et al., 2016a). Thirdly, challenge with *V. anguillarum* WT resulted in significant mortality in Dover sole larvae with an increased death rate noted following inoculation with a higher concentration (39% (1×10^6 CFU/mL) vs 50% (1×10^7 CFU/mL)). Although retrieved from a different fish species that is sea bass, adequate virulence hence was established. These results again underscore the complexity of fish species-specific virulence of *V. anguillarum*, impeding extrapolation of experimental challenge models across species and warranting further research.

Although efforts were and are still being made to understand the physiological changes during stress events (Pederzoli & Mola, 2016), research concerning welfare and pain awareness parameters in fish larvae remains practically nonexistent. Studies on predictive behavioural traits indicating severe suffering or imminent mortality are imperative and should allow to delineate humane endpoints for fish larvae. The individual housing of the larvae as is the case in the current experimental set-up, may be regarded as an aid in this research journey. In order to reduce the number of experimental animals used, a subsequent experiment with an increasing bacterial concentration was only performed when the lower bacterial titer did not generate sufficient mortality. In the third experiment, *V. anguillarum* WT was administered at a higher dose (1×10^7 CFU/mL) since only this strain showed pathogenic potential when administered at a lower concentration (1×10^6 CFU/mL). As no increased larval mortality was observed after inoculation with *V. anguillarum* HI610 and *V. harveyi* WT at 1×10^6 CFU/mL, both isolates were omitted in the third experiment.

Once the standardized challenge model was developed, the protective potential of two probiotic candidates was evaluated. Probiotics are usually defined as products which contain viable non-pathogenic micro-organisms able to confer health benefits to the host (FAO, 2016). The effectiveness of probiotic in terms of protection against bacterial pathogens especially was tested in juveniles and adult fish (reviewed in Verschuere et al., 2000 & Nayak, 2010) but also a small number of studies on marine fish larvae were performed. The protective potential of various probiotic candidates involving turbot larvae was evaluated in bottles (Gatesoupe, 1994) or tanks (Garcia de la Banda et al., 1992;

Gatesoupe, 1997). To our knowledge, only one study tested the protective potential of probiotic candidates in a challenge experiment using multi well plates (based on sea bass larvae, Schaeck et al., 2016b), hereby highlighting the significance of the present study on Dover sole larvae.

Different probiotics were tested for flatfish species, mainly focusing on turbot, with only a limited number of studies involving fish larvae (turbot (Huys et al., 2001; Hjelm et al., 2004; Planas et al., 2005; Munoz-Atienza et al., 2014), California halibut (*Paralichthys californicus*) (Zorilla et al., 2003), Dover sole (Avella et al., 2011), Senegalese sole (Makridis et al., 2008; Lobo et al., 2014). Considering sole species, Senegalese sole is widely studied and one probiotic *Shewanella putrefaciens* was put forward. Beneficial effects on growth, stress levels, onset of metamorphosis, intestinal flora and resistance against infection with *Photobacterium damselae* were found (reviewed in Tapia-Paniagua et al., 2012). For Dover sole, only one probiotic was described (*Enterococcus faecium*, Avella et al., 2011), modulating amongst others growth and cortisol levels (Palermo et al., 2011). However, the protective potential of this probiotic against challenge with a pathogenic agent was not evaluated. In this study *V. lentus* and *V. proteolyticus* were evaluated as probiotic candidates. *Vibrio lentus* was described as the causative agent of skin lesions and mortality in wild octopus (*Octopus vulgaris*) (Farto et al., 2003) but no pathogenic properties were observed in fish, following both intraperitoneal injection (Farto et al., 2003) or challenge via *Artemia* nauplii (Austin et al., 2005). Also in the present study no decreased survival of the larvae was observed, but due to the large width of the 95% CI, a negative impact on fish larval health may not be fully excluded. However, at 17 DAH a significant increase in standard body length of the larvae inoculated with *V. lentus* compared with the control group was noted. A beneficial growth effect has been correlated with the administration of probiotics via bioencapsulation to the live feed in fish larvae and juveniles (Gatesoupe, 1997; Carnevali et al., 2006) but no such effect has been described in marine larvae after probiotic treatment via the water. Increased growth rates due to probiotic administration were linked to the production of beneficial dietary compounds or digestive enzymes (Vine et al., 2006). The probiotic potential of *V. lentus* was previously tested in gnotobiotic sea bass larvae against *V. harveyi* WT, showing significantly decreased mortality rates (Schaeck et al., 2016b). In the present study, however, no such properties were seen in Dover sole larvae after challenge with *V. anguillarum* WT. No length measurements were performed by Schaeck et al. (2016a). *Vibrio proteolyticus* can induce mortality in *Artemia* cultures (Verschuere et al., 2000) but no such characteristics have been reported in fish. Also in this study, no increased mortality nor impaired larval growth was observed after administration of this isolate. Beneficial effects of diet supplementation with *V. proteolyticus* on protein degradation were noted (De Schrijver & Ollevier, 2000). However, the potential beneficial role of *V. proteolyticus* as a probiotic agent has not yet been described in aquatic organisms. This renders the present study the first in its kind although no positive effects, observed as

an increased survival after challenge with the pathogen or increased standard body length, were discerned.

To test the protective effect of the probiotic candidates during *V. anguillarum* WT infection, two routes of delivery, via the well water and via live feed (*Artemia* nauplii), were tested. Adding the probiotic candidate through the rearing water maximizes the exposure of the larvae before the start of exogenous feeding and during the first days, when the food intake is limited (Reitan et al., 1998; Vine et al., 2006). Furthermore, bath challenge may maximize the competitive advantage of added probiotics, as bacteria colonizing the intestines before first feeding may be able to persist amongst the autochthonous microflora (Hansen & Olafsen, 1999; Carnevali et al., 2006). Exogenous feeding was started at 6 DAH in Dover sole larvae and also the delivery of the probiotics via *Artemia* nauplii was studied. Delivery through bioencapsulation may be preferred in hatcheries where water exchange rates are high (Vine et al., 2006). For bioencapsulation, lower amounts of probiotic components are needed compared to when these are added to the water, rendering this practice more feasible and economically more interesting. One may presume that delivery through the feed results in the probiotic residing longer in the intestine and hence may increase the probiotic protective potential. In addition, it was described that colonization of the gut increases during exogenous feeding, hereby resembling the microflora of the livefood (Munro et al., 1994). Most probiotic studies focus only on one route of delivery, via live feed (e.g. Gatesoupe, 1994) or via the water (e.g. Makridis et al., 2008), underscoring the completeness of this study.

In addition to its possible value for many other applications, this experimental infection model for vibriosis constitutes a firm basis to evaluate the impact of (a)biotic components on larval health. The model is also to be regarded as a powerful tool for investigating the pathogenesis of *V. anguillarum* infections in Dover sole larvae, evaluating curative or preventive treatments and elucidating their mode(s) of action. In this study, probiotic candidates were selected in vitro and assessed for their protective potential against *V. anguillarum* challenge in vivo but also prebiotic treatments may be evaluated by means of this model. Indeed, although immunostimulating properties are allocated to prebiotics (Bricknell & Dalmo, 2005), limited research on the potential protective effect of prebiotics against challenge with a known pathogen has been performed in marine fish larvae (Skjermö and Bergh, 2004). Next to aquaculture related research, this model also renders biological research concerning the impact of environmental components (e.g. microplastics or algal toxins released during harmful algal blooms) on the health of Dover sole larvae possible by evaluating their susceptibility to disease agents.

6.6 ACKNOWLEDGEMENTS

Prof. dr. P. Simoens is gratefully acknowledged for critically reviewing the manuscript. Furthermore, the authors would like to thank Alain Le Breton for kindly providing the *V. anguillarum* WT and *V. harveyi* WT isolates and the Laboratory of Aquaculture & Artemia Reference Center (UGent) for *V. anguillarum* HI610. Wageningen Marine Research (the Netherlands) and Stichting Zeeschelp (the Netherlands) are gratefully acknowledged for providing the *S. solea* eggs. Marleen Foubert is thanked for her assistance in the bacterial strain identification based on 16S rRNA gene sequencing.

Table 6.1 - Overview of the survival (%) of Dover sole larvae during the development of the experimental infection model. Dover sole larvae were challenged at 10 DAH and survival was monitored until 17 DAH. In the three experiments, the pathogens were added in a final concentration of $1x10^5$ CFU/ml, $1x10^6$ CFU/ml and $1x10^7$ CFU/ml, respectively. The negative control group consisted of larvae with no bacterial cells added. Different letters (a & b) indicate statistically significant differences at p<0.0167 for experiment 1 and 2 and at p<0.05 for experiment 3.

	Treatment group	**Survival (%)**
Experiment 1 ($1x10^5$)	Negative control	91^a
	V. anguillarum HI610	86^a
	V. anguillarum WT	84^a
	V. harveyi WT	94^a
Experiment 2 ($1x10^6$)	Negative control	90^a
	V. anguillarum HI610	93^a
	V. anguillarum WT	61^b
	V. harveyi WT	86^a
Experiment 3 ($1x10^7$)	Negative control	92^a
	V. anguillarum WT	50^b

*Table 6.2 - Overview of the survival (%) and mean standard body length (µm) of Dover sole larvae at 17 DAH, supplemented at 4, 6 and 8 DAH with the probiotic candidates (*V. proteolyticus *and* V. lentus*). The negative control group consisted of larvae with no bacterial cells added. Different letters (a & b) indicate statistically significant differences at p<0.0167. n= number of larvae included to calculate the mean standard body length.*

	Treatment group	Survival (%)	Mean standard body length (µm)
Experiment 1	Negative control	76[a]	4505.36[a]
	V. proteolyticus	74[a]	4346.99[a]
Experiment 2	Negative control	89[a]	5000.06[b]
	V. lentus	88[a]	5570.49[a]

*Table 6.3 – Overview of the survival (%) at 21 DAH of Dover sole larvae supplemented at 4, 6 and 8 DAH with the probiotic candidates (*V. proteolyticus *and* V. lentus*) via the water (experiment 1) or the feed (experiment 2) followed by challenge with* V. anguillarum *WT at 10 DAH. The negative control group consisted of larvae with no bacterial cells added. A positive control group comprised larvae challenged with* V. anguillarum *at 10 DAH, without probiotic supplementation. Different letters (a & b) indicate statistically significant differences at p<0.0167.*

	Treatment group	**Survival (%)**
Experiment 1 (water)	Negative control	80[b]
	V. anguillarum WT	35[a]
	V. proteolyticus + V. anguillarum WT	37[a]
	V. lentus + V. anguillarum WT	34[a]
Experiment 2 (feed)	Negative control	72[a]
	V. anguillarum WT	52[a]
	V. proteolyticus + V. anguillarum WT	45[b]
	V. lentus + V. anguillarum WT	49[a]

6.7 REFERENCES

Arijo, S., Chabrillon, M., Diaz-Rosales, P., Rico, R.M., Martinez-Manzanares, E., Balebona, M.C., Toranzo, A.E. & Morinigo, M.A. (2005) Bacteria isolated from outbreaks affecting cultured sole, *Solea senegalensis* (Kaup). *Bulletin of the European Association of Fish Pathologists,* **25,** 148-154.

Avella, M.A., Olivotto, I., Silvi, S., Ribecco, C., Cresci, A., Palermo, F., Polzonetti, A. & Carnevali, O. (2011) Use of Enterococcus faecium to improve common sole (*Solea solea*) larviculture. *Aquaculture,* **315,** 384-393.

Austin, B. & Austin, D.A. (2007) *Bacterial fish pathogens: disease of farmed and wild fish*. Fourth edition, Praxis Publishing Ltd, Chichester, UK, p552

Austin, B. & Austin, D.A. (2012) *Bacterial Fish Pathogens: disease of farmed and wild fish*. Fifth edition. Springer Dordrecht

Baele, M., Devriese, L.A. & Haesebrouck, F. (2001) *Lactobacillus agilis* is an important component of the pigeon crop flora. *Journal of Applied Microbiology,* **91,** 488-491.

Bergh, O., Hjeltnes, B. & Skiftesvik, A.B. (1997) Experimental infection of turbot *Scophthalmus maximus* and halibut *Hippoglossus hippoglossus* yolk sac larvae with *Aeromonas salmonicida* subsp *salmonicida*. *Diseases of Aquatic Organisms,* **29,** 13-20.

Bjørndal, T., Guillen, J. & Imsland, A. (2016) The potential of aquaculture sole production in Europe: Production costs and markets. *Aquaculture Economics & Management,* **20,** 109-129.

Bricknell, I. & Dalmo, R.A. (2005) The use of immunostimulants in fish larval aquaculture. *Fish & Shellfish Immunology,* **19,** 457-472.

Cabello, F.C. (2006) Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environmental Microbiology,* **8,** 1137-1144.

Carnevali, O., De Vivo, L., Sulpizio, R., Gioacchini, G., Olivotto, I., Silvi, S. & Cresci, A. (2006) Growth improvement by probiotic in European sea bass juveniles (*Dicentrarchus labrax*, L.), with particular attention to IGF-1, myostatin and cortisol gene expression. *Aquaculture,* **258,** 430-438.

Coenye, T., Falsen, E., Vancanneyt, M., Hoste, B., Govan, J.R.W., Kersters, K. & Vandamme, P. (1999) Classification of *Alcaligenes faecalis*-like isolates from the environment and human clinical samples as *Ralstonia gilardii* sp. nov. *International Journal of Systematic Bacteriology,* **49,** 405-413.

Cui, L., Xu, W., Ai, Q., Wang, D. & Mai, K. (2012) Effects of dietary chitosan oligosaccharide complex with rare earth on growth performance and innate immune response of turbot, *Scophthalmus*

maximus L. *Aquaculture Research,* **44**, 683–690.

D'alvise, P.W., Lillebo, S., Wergeland, H.I., Gram, L. & Bergh, O. (2013) Protection of cod larvae from vibriosis by *Phaeobacter* spp.: A comparison of strains and introduction times. *Aquaculture,* **384,** 82-86.

De, B.C., Meena, D.K., Behera, B.K., Das, P., Das Mohapatra, P.K. & Sharma, A.P. (2014) Probiotics in fish and shellfish culture: immunomodulatory and ecophysiological responses. *Fish Physiology and Biochemistry,* **40,** 921-971.

De La Banda, I.G., Lobo, C., Leon-Rubio, J.M., Tapia-Paniagua, S., Balebona, M.C., Morinigo, M.A., Moreno-Ventas, X., Lucas, L.M., Linares, F., Arce, F. & Arijo, S. (2010) Influence of two closely related probiotics on juvenile Senegalese sole (*Solea senegalensis*, Kaup 1858) performance and protection against *Photobacterium damselae* subsp *piscicida*. *Aquaculture,* **306,** 281-288.

De Schrijver, R. & Ollevier, F. (2000) Protein digestion in juvenile turbot (*Scophthalmus maximus*) and effects of dietary administration of *Vibrio proteolyticus*. *Aquaculture,* **186,** 107-116.

De Swaef, E., Demeestere, K., Boon, N., Van Den Broeck, W., Haesebrouck, F. & Decostere, A. (2017) Development of a reliable experimental set-up for Dover sole larvae *Solea solea* L. and exploring the possibility of implementing this housing system in a gnotobiotic model. *Research in Veterinary Science,* **115,** 418-424.

Declercq, A.M., Boyen, F., Van Den Broeck, W., Bossier, P., Karsi, A., Haesebrouck, F. & Decostere, A. (2013) Antimicrobial susceptibility pattern of *Flavobacterium columnare* isolates collected worldwide from 17 fish species. *Journal of Fish Diseases,* **36,** 45-55.

Diaz-Rosales, P., Arijo, S., Chabrillon, M., Alarcon, F.J., Tapia-Paniagua, S.T., Martinez-Manzanares, E., Balebona, M.C. & Morinigo, M.A. (2009) Effects of two closely related probiotics on respiratory burst activity of Senegalese sole (*Solea senegalensis*, Kaup) phagocytes, and protection against *Photobacterium damselae* subsp *piscicida*. *Aquaculture,* **293,** 16-21.

Dierckens, K., Rekecki, A., Laureau, S., Sorgeloos, P., Boon, N., Van Den Broeck, W. & Bossier, P. (2009) Development of a bacterial challenge test for gnotobiotic sea bass (*Dicentrarchus labrax*) larvae. *Environmental Microbiology,* **11,** 526-533.

FAO (2016) Food and Agriculture Organization of the United Nations, www.fao.org. Accessed 4 May 2017

Farto, R., Armada, S.P., Montes, M., Guisande, J.A., Perez, M.J. & Nieto, T.P. (2003) Vibrio lentus associated with diseased wild octopus (*Octopus vulgaris*). *Journal of Invertebrate Pathology,* **83,** 149-

156.

Ferraresso, S., Bonaldo, A., Parma, L., Cinotti, S., Massi, P., Bargelloni, L. & Gatta, P.P. (2013) Exploring the larval transcriptome of the common sole (*Solea solea* L.). *Bmc Genomics,* **14**.

Frans, I., Dierckens, K., Crauwels, S., Van Assche, A., Leisner, J., Larsen, M.H., Michiels, C.W., Willems, K.A., Lievens, B., Bossier, P. & Rediers, H. (2013) Does Virulence Assessment of *Vibrio anguillarum* Using Sea Bass (*Dicentrarchus labrax*) Larvae Correspond with Genotypic and Phenotypic Characterization? *Plos One,* **8**.

Gatesoupe, F.J. (1994) Lactic-Acid Bacteria Increase the Resistance of Turbot Larvae, *Scophthalmus Maximus*, against Pathogenic *Vibrio*. *Aquatic Living Resources,* **7,** 277-282.

Gatesoupe, F.J. (1997) Siderophore production and probiotic effect of *Vibrio* sp. associated with turbot larvae, *Scophthalmus maximus*. *Aquatic Living Resources,* **10,** 239-246.

Hansen, G.H. & Olafsen, J.A. (1999) Bacterial interactions in early life stages of marine cold water fish. *Microbial Ecology,* **38,** 1-26.

Hjelm, M., Bergh, O., Riaza, A., Nielsen, J., Melchiorsen, J., Jensen, S., Duncan, H., Ahrens, P., Birkbeck, H. & Gram, L. (2004) Selection and identification of autochthonous potential probiotic bacteria from turbot larvae (*Scophthalmus maximus*) rearing units. *Systematic and Applied Microbiology,* **27,** 360-371.

Huys, L., Dhert, P., Robles, R., Ollevier, F., Sorgeloos, P. & Swings, J. (2001) Search for beneficial bacterial strains for turbot (*Scophthalmus maximus* L.) larviculture. *Aquaculture,* **193,** 25-37.

Li, X., Defoirdt, T., Yang, Q., Laureau, S., Bossier, P. & Dierckens, K. (2014) Host-induced increase in larval sea bass mortality in a gnotobiotic challenge test with *Vibrio anguillarum*. *Diseases of Aquatic Organisms,* **108,** 211-216.

Lobo, C., Moreno-Ventas, X., Tapia-Paniagua, S., Rodriguez, C., Morinigo, M.A. & De La Banda, I.G. (2014) Dietary probiotic supplementation (*Shewanella putrefaciens* Pdp11) modulates gut microbiota and promotes growth and condition in Senegalese sole larviculture. *Fish Physiology and Biochemistry,* **40,** 295-309.

Makridis, P., Martins, S., Reis, J. & Dinis, M.T. (2008) Use of probiotic bacteria in the rearing of Senegalese sole (*Solea senegalensis*) larvae. *Aquaculture Research,* **39,** 627-634.

Manfrin, A., Doimi, M., Antonetti, P,. Delgado Montero, M.L., Qualtieri, K., Rampazzo, E., Vascellari, M., Mutinelli, F., Melchiotti, E., Bozza, M.A., Selli, L. & Bovo, G. (2003) Primo isolamento di *Vibrio*

anguillarum sierotipo O 1 da sogliola (*Solea solea*, L.) in Itali/ First isolation of *Vibrio anguillarum* O1 serotype from sole (*Solea solea*) in Italy. *Bollettino Societa Italiana di Patologia Ittica* **37,** 12-17.

Munoz-Atienza, E., Araujo, C., Magadan, S., Hernandez, P.E., Herranz, C., Santos, Y. & Cintas, L.M. (2014) In vitro and in vivo evaluation of lactic acid bacteria of aquatic origin as probiotics for turbot (*Scophthalmus maximus* L.) farming. *Fish & Shellfish Immunology,* **41,** 570-580.

Munro, P.D., Barbour, A. & Birkbeck, T.H. (1994) Comparison of the Gut Bacterial-Flora of Start-Feeding Larval Turbot Reared under Different Conditions. *Journal of Applied Bacteriology,* **77,** 560-566.

Munro, P.D., Barbour, A. & Birkbeck, T.H. (1995) Comparison of the Growth and Survival of Larval Turbot in the Absence of Culturable Bacteria with Those in the Presence of *Vibrio Anguillarum*, *Vibrio Alginolyticus*, or a Marine *Aeromonas* Sp. *Applied and Environmental Microbiology,* **61,** 4425-4428.

Muroga, K., Yasunobu, H., Okada, N. & Masumura, K. (1990) Bacterial Enteritis of Cultured Flounder *Paralichthys Olivaceus* Larvae. *Diseases of Aquatic Organisms,* **9,** 121-125.

National Committee for Clinical Laboratory Standards (1997) Performance standards for analysis antimicrobial disk susceptibility tests. Approved standard M2-A6, Wayne, **19**, 1-7.

Nayak, S.K. (2010) Probiotics and immunity: A fish perspective. *Fish & Shellfish Immunology,* **29,** 2-14.

Novriadi, R. (2016) Vibriosis in aquaculture. *Omni-Akuatika,* **12,** 1-12.

Palermo, F.A., Mosconi, G., Avella, M.A., Carnevali, O., Verdenelli, M.C., Cecchini, C. & Polzonetti-Magni, A.M. (2011) Modulation of cortisol levels, endocannabinoid receptor 1A, proopiomelanocortin and thyroid hormone receptor alpha mRNA expressions by probiotics during sole (*Solea solea*) larval development. *General and Comparative Endocrinology,* **171,** 293-300.

Paolini, A., Magi, G.E., Gennari, L., Egidi, C., Torresi, M., Vallerani, M., Berti, M., Pavone, A. & Giorgetti, G. (2010). Severe mortality in common sole (*Solea solea*) at post-weaning stage following environmental stress. *Bulletin of the European Association of Fish Pathologists,* **30**, 160-169.

Pederzoli, A. & Mola, L. (2016) The early stress responses in fish larvae. *Acta Histochem,* **118,** 443-449.

Planas, M., Perez-Lorenzo, M., Vazquez, J.A. & Pintado, J. (2005) A model for experimental infections with *Vibrio (Listonella) anguillarum* in first feeding turbot (*Scophthalmus maximus* L.) larvae under hatchery conditions. *Aquaculture,* **250,** 232-243.

Reitan, K.I., Natvik, C.M. & Vadstein, O. (1998) Drinking rate, uptake of bacteria and microalgae in turbot larvae. *Journal of Fish Biology,* **53,** 1145-1154.

Ringø, E., Olsen, R.E., Gifstad, T.O., Dalmo, R.A., Amlund, H., Hemre, G.I. & Bakke, A.M. (2010) Prebiotics in aquaculture: a review. *Aquaculture Nutrition,* **16,** 117-136.

Ringø, E., Olsen, R.E., Vecino, J.L.G., Wadsworth, S. & Song, S.K. (2012) Use of immunostimulants and nucleotides in aquaculture: a review. *Journal of Marine Science: Research and Development,* **2**, 104.

Rodgers, P.E., Read, A. & Meun, G. (2005) Benefits of Responsible Fishing: The impact of an innovative trial of voluntary restraint. Report for the EU Directorate-General for Fisheries, Contract No. 2003/C 115/08-17, CFER, Lincoln.

Romero, J., Feijoo, C.G. & Navarrete, P. (2012) Antibiotics in Aquaculture – Use, Abuse and Alternatives. In: Carvalho ED, David GS, Silva RJ (eds) *Health and Environment in Aquaculture*. InTech, Rijeka, 159-198.

Rønneseth, A., Castillo, D., D'alvise, P., Tonnesen, O., Haugland, G., Grotkjaer, T., Engell-Sorensen, K., Norremark, L., Bergh, O., Wergeland, H.I. & Gram, L. (2017) Comparative assessment of *Vibrio* virulence in marine fish larvae. *Journal of Fish Diseases,* **40,** 1373-1385.

Samuelsen, O.B., Bergh, O. & Ervik, A. (2003) Pharmacokinetics of florfenicol in cod *Gadus morhua* and *in vitro* antibacterial activity against *Vibrio anguillarum*. *Diseases of Aquatic Organisms,* **56,** 127-133.

Sandlund, N. & Bergh, O. (2008) Screening and characterisation of potentially pathogenic bacteria associated with Atlantic cod *Gadus morhua* larvae: bath challenge trials using a multidish system. *Diseases of Aquatic Organisms,* **81,** 203-217.

Sandlund, N., Rodseth, O.M., Knappskog, D.H., Fiksdal, I.U. & Bergh, O. (2010) Comparative susceptibility of turbot, halibut, and cod yolk-sac larvae to challenge with *Vibrio* spp. *Diseases of Aquatic Organisms,* **89,** 29-37.

Schaeck, M., De Swaef, E., Van Den Broeck, W., Van Nevel, S., Boon, N., De Geyter, N., Morent, R., Demeestere, K., Duchateau, L., Coulombet, C., Haesebrouck, F. & Decostere, A. (2016a) Germ-free sea bass *Dicentrarchus labrax* larval model: a valuable tool in the study of host-microbe interactions. *Diseases of Aquatic Organisms,* **117,** 177-185.

Schaeck, M., Duchateau, L., Van Den Broeck, W., Van Trappen, S., De Vos, P., Coulombet, C., Boon, N., Haesebrouck, F. & Decostere, A. (2016b) *Vibrio lentus* protects gnotobiotic sea bass (*Dicentrarchus labrax* L.) larvae against challenge with *Vibrio harveyi*. *Veterinary Microbiology,* **185,** 41-48.

Seljestokken, B., Bergh, O., Melingen, G.O., Rudra, H., Olsen, R.H. & Samuelsen, O.B. (2006) Treating experimentally induced vibriosis (*Listonella anguillarum*) in cod, *Gadus morhua* L., with florfenicol. *Journal of Fish Diseases,* **29,** 737-742.

Silva, Y.J., Costa, L., Pereira, C., Mateus, C., Cunha, A., Calado, R., Gomes, N.C.M., Pardo, M.A., Hernandez, I. & Almeida, A. (2014) Phage Therapy as an Approach to Prevent *Vibrio anguillarum* Infections in Fish Larvae Production. *Plos One,* **9**.

Skjermö, J. & Bergh, O. (2004) High-M alginate immunostimulation of Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae using Artemia for delivery, increases resistance against vibriosis. *Aquaculture,* **238,** 107-113.

Smet, A., Flahou, B., D'herde, K., Vandamme, P., Cleenwerck, I., Ducatelle, R., Pasmans, F. & Haesebrouck, F. (2012) *Helicobacter heilmannii* sp. nov., isolated from feline gastric mucosa. *International Journal of Systematic and Evolutionary Microbiology,* **62,** pg 229.

Sorgeloos, P., Food, Nations, A.O.O.T.U. & Landbouwwetenschappen, R.T.G.F.V.D. (1986) *Manual for the culture and use of brine shrimp artemia in aquaculture,* State University of Ghent, Faculty of Agriculture.

Tapia-Paniagua, S.T., Diaz-Rosales, P., Leon-Rubio, J.M., De La Banda, I.G., Lobo, C., Alarcon, F.J., Chabrillon, M., Rosas-Ledesma, P., Varela, J.L., Ruiz-Jarabo, I., Arijo, S., Esteban, M.A., Martinez-Manzanares, E., Mancera, J.M., Balebona, M.C. & Morinigo, M.A. (2012) Use of the probiotic *Shewanella putrefaciens* Pdp11 on the culture of Senegalese sole (*Solea senegalensis*, Kaup 1858) and gilthead seabream (*Sparus aurata* L.). *Aquaculture International,* **20,** 1025-1039.

Thompson, F.L., Iida, T. & Swings, J. (2004) Biodiversity of vibrios. *Microbiology and Molecular Biology Reviews,* **68,** 403-+.

Tinh, N.T.N., Dierckens, K., Sorgeloos, P. & Bossier, P. (2008) A review of the functionality of probiotics in the larviculture food chain. *Marine Biotechnology,* **10,** 1-12.

Toranzo, A.E., Magarinos, B. & Romalde, J.L. (2005) A review of the main bacterial fish diseases in mariculture systems. *Aquaculture,* **246,** 37-61.

Touraki, M., Niopas, I. & Karagiannis, V. (2012) Treatment of vibriosis in European sea bass larvae, *Dicentrarchus labrax* L., with oxolinic acid administered by bath or through medicated nauplii of *Artemia franciscana* (Kellogg): efficacy and residual kinetics. *Journal of Fish Diseases,* **35,** 513-522.

Verschuere, L., Heang, H., Criel, G., Sorgeloos, P. & Verstraete, W. (2000) Selected bacterial strains protect *Artemia* spp. from the pathogenic effects of *Vibrio proteolyticus* CW8T2. *Applied and Environmental Microbiology,* **66,** 1139-1146.

Vine, N.G., Leukes, W.D. & Kaiser, H. (2006) Probiotics in marine larviculture. *Fems Microbiology Reviews,* **30,** 404-427.

Willis, C. (2000) Antibiotics in the food chain: their impact on the consumer. *Reviews in Medical Microbiology,* **11,** 153-160.

Zorrilla, I., Arijo, S., Chabrillon, M., Diaz, P., Martinez-Manzanares, E., Balebona, M.C. & Morinigo, M.A. (2003) *Vibrio* species isolated from diseased farmed sole, *Solea senegalensis* (Kaup), and evaluation of the potential virulence role of their extracellular products. *Journal of Fish Diseases,* **26,** 103-108.

Chapter 7 THE IMPACT OF DIETARY SUPPLEMENTATION OF MANNANOLIGOSACCHARIDE AND ALGINIC ACID IN DOVER SOLE *SOLEA SOLEA* LARVAE, INCLUDING ON THE PROTECTION AGAINST *VIBRIO ANGUILLARUM* CHALLENGE

7.1 ABSTRACT

Dover sole (*Solea solea*) larval production performance is affected by limited knowledge on the nutritional requirements as well as arduous disease control. For the latter, alternative disease prevention and control measures, including prebiotics, are extensively investigated. The present study aimed at assessing the impact of dietary supplementation of two prebiotic candidates, mannanoligosaccharide and alginic acid, in Dover sole larvae. Although increased fish health and performance were reported for other fish species, no increased larval growth nor shifted proliferation and apoptosis rate of the intestinal epithelium was observed in Dover sole larvae following 0.2% or 0.4% mannanoligosaccharide supplementation. In contrast, administration of 0.4% alginic acid resulted in a decreased standard body length. No difference in survival was observed following administration of both compounds. For the first time, the protective potential against pathogen challenge with *Vibrio anguillarum* following supplementation with prebiotic candidates was determined in Dover sole larvae. No difference in survival at 21 days after hatching was discerned in the groups of larvae supplemented with 0.2% and 0.4% mannanoligosaccharide or 0.4% alginic acid followed by pathogen challenge compared to the non-supplemented challenged larvae. However, the larvae supplied with 0.2% alginic acid followed by challenge displayed a lower survival in comparison with the challenged larvae without any supplementation. This might reveal a possible negative impact of alginic acid supplementation on Dover sole larvae, although survival and therefore sample sizes were limited due to low larval quality in this experiment. The lower survival observed following pathogen challenge highlights the importance of including both direct as well as indirect parameters to evaluate the harmlessness of new compounds.

7.2 INTRODUCTION

Dover sole (*Solea solea*) is recognized by restaurant owners as being an excellent ingredient for high end gastronomy and has already been favorited by European consumers for a long time (Rodgers et al., 2005; Bjørndal et al., 2016). Together with its high market value and the general need of fish farmers to diversify their operations (Lane et al., 2014; European Union DIVERSIFY, 2016), this resulted in a renewed interest in sole aquaculture during recent years, both at research (Lund, 2007; Ferraresso et al., 2013) and commercial level (Bjørndal et al, 2015; 2016; FAO, 2018).

As for many other species, production performance is affected by low feed conversion ratios, inadequate growth rates and arduous disease control, limiting the success of this species in aquaculture. Especially for the larviculture phase, mortality due to disease is still an important bottleneck, with vibriosis being one of the most important bacterial diseases worldwide (Austin & Austin, 2012). One of the main causative agents of vibriosis is *Vibrio anguillarum*, affecting more than 90 aquatic organisms including Dover sole (Palazzi et al., 2006; Paolini et al., 2010; Hickley & Lee, 2018). The use of various antimicrobial agents as prophylactic as well as therapeutic measures reduced the impact of bacterial diseases in the past. However, due to the environmental and both human and animal health risks (Skjermo & Vadstein, 1999; Burridge et al., 2010), there is an increasing demand for more sustainable control measures, resulting in a large expansion of the research involving alternatives (Bricknell & Dalmo, 2005; Skjermo et al., 2015; Ringø et al., 2016; Vadstein et al., 2018).

During the last decade, one of the largely studied alternatives in fish are prebiotics, not only for their role in improving disease resistance (as an immunostimulant) but also as a functional dietary supplement as reviewed by several authors (Bricknell & Dalmo, 2005; Ringø et al., 2014; Song et al., 2014; Torrecillas et al., 2014; Hoseinifar et al., 2015). Many different components were already tested, but the most commonly used prebiotics in fish are inulin and various oligosaccharides (fructooligosaccharide (FOS), mannanoligosaccharide (MOS), galactooligosaccharides (GOS)) (Ringø et al., 2014). Amongst others, prebiotics were described to positively influence the gastrointestinal tract, resulting in higher survival, increased growth performance, enzyme activity and changed gastrointestinal tract morphology (Dimitroglou et al., 2009; 2010; 2011; Ringø et al., 2010) for various fish families and species (reviewed in Ringø et al., 2014; Hoseinifar et al., 2015). Although many studies described advantageous effects of prebiotic administration in adult fish, the amount of studies focusing on fish larvae is much more limited.

Mannanoligosaccharide derived from the cell wall of baker's yeast (*Saccharomyces cerevisiae*), has been used for many years as a prebiotic component in terrestrial animals and recently its effectiveness in aquaculture was established (Torrecillas et al 2007; 2011; Dimitroglou et al., 2009; 2010). Indeed, in

adults, MOS increased gut health by more efficient food conversion and improved innate immunity (reviewed in Ringø et al., 2010; Carbone & Faggio, 2016). Furthermore, dietary MOS supplementation was linked to reduced mortality following challenge with several fish pathogens (*Aeromonas* spp. (Dimitroglou et al., 2009), *Vibrio* spp. (Torrecillas et al., 2007, 2011; 2012; Dimitroglou et al., 2009; Rodriguez-Estrada et al., 2009) and *Photobacterium damselae* (Dimitroglou et al., 2011)). However, research involving marine fish larvae remains limited to white seabream *Diplodus sargus* (Dimitroglou et al., 2010) and cobia *Rachycentron canadum* (Salze et al., 2008). For neither of the two species, the effect of MOS supplementation on survival following pathogen challenge was investigated.

Recently, the use of prebiotics derived from the aquatic environment was favored over components of terrestrial origin (Romero et al, 2012). In this respect, various currently adopted prebiotic candidates are derived from seaweeds (Kakuta, 2004; Caipang et al., 2011; Morales-Lange et al., 2015; Vatsos and Rebours, 2015). One of the most promising components are alginates; polysaccharides found in the cell wall of several brown seaweed species including the genera *Laminaria, Macrocystis* and *Fucus* (Peddie et al., 2002). Alginates were administered via the food in different compounds (a.o alginic acid (AA), sodium alginate, highM alginates) and are also known as the active ingredient of the commercial food supplement Ergosan ™ (e.g. Gioacchini et al., 2008; Akbari et al., 2015).

The aim of this study was to assess the impact of two potentially prebiotic components, MOS and AA, on larval (gut) health in Dover sole. Larval growth and both proliferation as well as apoptosis rate of the intestinal epithelium were included as parameters. Furthermore, for the first time, the protective potential of their supplementation following pathogen challenge with *V. anguillarum* was determined.

7.3 MATERIAL AND METHODS

All experiments were approved by the Ethical Committee of the Faculty of Veterinary Medicine and Bio-engineering Sciences, Ghent University (No. EC2017/31).

7.3.1 Larval rearing

Naturally spawned Dover sole eggs were obtained from Stichting Zeeschelp (Kamperland, the Netherlands) and transported in natural seawater of 32g/L after removal of dead eggs. Upon arrival, eggs were acclimatized with autoclaved artificial seawater (AASW) of 34 g/L (Instant Ocean, Aquarium Systems, Mentor, Ohio) and further incubated herein under aeration.

The eggs and larvae were housed according to the protocol as described in De Swaef et al. (2017) with slight modifications. Eggs and newly hatched larvae were transferred to glass bottles filled with 400 mL AASW at 16 ± 1 °C without egg disinfection. These bottles were aerated with filtered (0.2 µm, Sartorius AG, Göttingen, Germany) sterile air and each bottle contained approximately 600 eggs. Two

days after hatching (DAH), larvae were placed individually in sterile polystyrene 24-well tissue culture plates (Greiner Bio-One, Frickenhausen, Germany), each well containing approximately 2 mL AASW. The larvae were fed ad libitum with *Artemia* nauplii (Sep-Art Artemia; INVE Aquaculture NV, Belgium) every other day, starting from 5 DAH onwards, except when indicated otherwise. Half of the well water was replaced every other day (prior to feeding) and all larvae were subjected to a circadian rhythm of 9 h light and 15 h darkness (De Swaef et al., 2017).

7.3.2 Bacterial isolate

Bacterial cultivation was performed according to De Swaef et al. (2018). *Vibrio anguillarum* was cultured on marine agar (MA, Scharlab S.L., Sentmenat, Spain) and incubated for 48 h at 18 °C, followed by cultivation in tryptic soy broth (Becton, Dickinson and Company, New Jersey, USA) supplemented with 1.5% NaCl for 24 h at 18 °C. Cells were harvested by centrifugation (3500 rpm for 10 min) and the resulting pellet was washed twice with AASW and subsequently resuspended in AASW. Optical densities were determined using an ATB 1550 densitometer (BioMérieux, Marcy-l'Etoile, France). Bacterial titres were verified by making a tenfold dilution series in triplicate on MA plates, prior to administration.

7.3.3 *Artemia* bioencapsulation

Artemia cysts were incubated at a concentration of 1.2 g/L at 28°C for 24h. Next, newly hatched *Artemia* nauplii were enriched with MOS (Biomos, Alltech, Klerken-Houthulst, Belgium) or AA (alginic acid sodium salt, ref.180947, Sigma-Aldrich, Diegem, Belgium) at a dose of 20mg/L or 40mg/L for minimally 6 h at 18 °C. Immediately following enrichment, the *Artemia* were rinsed with AASW to remove any remaining MOS or AA before being fed to the larvae.

7.3.4 Experimental design

The prebiotic potential of AA and MOS was tested in two separate (in time) experiments. For each experimental trial, Dover sole eggs from one single batch were used. Both experiments included six groups. Two groups of 156 larvae were provided daily with *Artemia* enriched with the prebiotic candidate (AA or MOS) from 4 to 9 DAH at two concentrations (0.2% and 0.4%). In addition, two groups of 96 larvae were fed with *Artemia* enriched with the prebiotic candidates in the same two concentrations, followed by a larval challenge with *V. anguillarum* WT at a final concentration of 1×10^7 colony forming units/mL at 10 DAH (De Swaef et al., 2018). A negative control group was included in which larvae underwent the same physical handling and water exchanges but without the addition of bacterial cells or enriched *Artemia*. A sixth group (positive control) was inoculated with *V. anguillarum* WT at 10 DAH without being previously administered a prebiotic candidate. All groups were maintained until 21 DAH.

To assess the survival, in each group 96 larvae divided over four 24-well plates were monitored until 21 DAH. The standard body length of 20 remaining larvae per treatment at 21 DAH was measured using an Olympus SZX7 stereomicroscope and cell D software (Soft imaging system, Olympus NV, Bergen, Belgium). For immunohistological examination, six larvae of the negative control group and the two groups provided with *Artemia* enriched with 0.2% and 0.4% of the prebiotic candidate were sampled at the end of the prebiotic supplementation period (10 DAH). Prior to sampling, larvae were sacrificed by immersion in an overdose of MS 222 (tricaine methanesulfonate, Sigma-Aldrich, Diegem, Belgium).

7.3.5 Immunohistochemistry

Larvae were fixed in 4% formadehyde for at least one week, followed by embedding in paraffin (Qpath paraffin, VWR, Oud-Heverlee, Belgium) and transversally sectioned at 5 µm. Larvae that were not perfectly transverse aligned, were removed prior to sectioning. Foregut sections were obtained starting from 10 µm caudally from the oesophagus and 12 sections were alternately intercepted for proliferation and apoptosis determination of enterocytes, ensuring six sections per larva per staining. The foregut was defined as the intestinal section immediately succeeding the oesophagus whereas the second transverse section of the intestine present on the same slide was delineated as midgut.

To determine the proliferation rate of enterocytes, anti-proliferating cell nuclear antigen (PCNA) antibody was employed. Following deparaffinization and rehydration, sections were treated for heat-induced epitope retrieval (HIER) in citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked in Dako REAL Peroxidase Blocking Solution (ref. S2023, DAKO, Diegem, Belgium) for 5 min. Subsequently, sections were treated with Mouse monoclonal PCNA antibody [PC-10] (ref. ab29, Abcam, Cambridge, UK) for 30 min, followed by washing with wash buffer 1:10 diluted (ref. S3006, DAKO, Diegem, Belgium). Next, the PC-10 antibody was captured with the labeled polymer- HRP anti-mouse (ref. K4007, DAKO, Diegem, Belgium), followed by incubation within 3, 39-diaminobenzidine (DAB)+ substrate buffer (ref K3468, DAKO, Diegem, Belgium) for 5 min. Tissue sections were counterstained with haematoxylin, followed by dehydration and mounting of the sections.

Cell apoptosis was detected by using Terminal deoxynucleotidyl transferase dUTP nick end labelling. After deparaffinization and rehydration, sections were incubated for 25 min at 37 °C with proteinase K (ref S3004, DAKO, Carpinteria, USA). Next, DNA labeling was performed using the In Situ Cell Death Detection Kit, Fluorescein (ref. 11684795910, Roche) according to the manufacturer instructions, to detect and quantify apoptotic cell death at the single-cell level by fluorescence microscopy. Cell nuclei were counterstained with Hoechst stain (blue).

Images were acquired using a Leica DMRB light and fluorescence microscope (Leica Microsystems, Diegem, Belgium). The proliferative and apoptotic indices were determined according to the

percentage of positive cells in a total of 100 cells assessed in the foregut and midgut sections. To count the PCNA-positive nuclei of enterocytes, scientific image analysis software (Leica LAS software) was used. The original image was divided into colour channels, which were manually chosen by the operator as DAB-stained positive nuclei. To assure objective assessment of the histological parameters, samples were blinded beforehand. Histological sections with the presence of tissue from the oesophagus, poor quality or uneven staining, were removed from the analysis.

7.3.6 Statistics

Survival percentages and length measurements were compared using analysis of variance with normal distribution assumption, which was tested by the Shapiro-Wilks test. All analyses were performed using SAS version 6.4. The global significance level of 5% was used, but the multiple comparisons of each treatment with the negative and the positive control were adjusted by Bonferroni's method. Therefore, the comparison wise significance level was set at 0.05/2 = 0.025. The proliferative and apoptotic indices were calculated per larvae by determining the mean value. To evaluate the effect of treatment (control vs MOS 0.2% or MOS 0.4%) within location (foregut or midgut), analysis of variance with normal distribution assumption (tested by the Shapiro-Wilks test) was used separately for each location using the Bonferroni correction for multiple comparisons adjustment.

7.4 RESULTS

7.4.1 Survival

In the first experiment, no significant differences in survival at 21 DAH were observed between the negative control group (49%) and the larvae supplemented with 0.2% (47%, p-value = 0.7486) or 0.4% AA (42%, p-value = 0.2697). Furthermore, following *V. anguillarum* challenge, a significant decrease in survival was discerned between the positive control group (36%) and the larvae supplemented with 0.2% AA (16%, p-value = 0.0044). A negative trend in survival (21%) at 21 DAH was observed in the group supplemented with 0.4% AA followed by the pathogen challenge compared with the positive control group (p-value = 0.0252) (Table 7.2).

In the second experiment, no significant differences in survival at 21 DAH were noted between the negative control group (53%) and the larvae supplemented with 0.2% MOS (66%, p-value = 0.0916) or 0.4% MOS (53%, p-value = 1.0000). Also following *V. anguillarum* challenge, no significant differences were noted between the survival of the positive control group (15%) and the larvae supplemented with 0.2% (17%, p-value = 0.7698) and 0.4% MOS (16%, p-value = 0.8836) (Table 7.2).

Standard body length

Sample sizes were deviant from the predetermined numbers in the larval groups challenged following supplementation with 0.2% (n = 3) and 0.4% AA (n = 6) as well as in both the groups of larvae challenged following supplementation with 0.2% (n = 4) and 0.4% MOS (n = 5) and the corresponding positive control group (n = 4) (Table 7.2).

No significant differences in standard body length between the negative control and larvae supplemented with 0.2% AA was noted (p-value = 0.2161). However, a significant decrease in standard body length was observed following 0.4% AA supplementation compared with the negative control (p-value = 0.0002). Furthermore, a significant difference between the positive control and larvae supplemented with 0.2% AA (p-value = 0.1638) followed by pathogen challenge was noticeable. No significant negative impact on standard body length was discerned in the group supplemented with 0.4% AA followed by the pathogen challenge compared with the positive control (p-value = 0.0147) (Table 7.2).

Following MOS supplementation, no significant differences in standard body length were observed at 21 DAH between any of the groups (all p-values > 0.200) (Table 7.2).

7.4.2 Immunohistochemistry

The resulting PCNA and TUNEL indices are summarized in table 1. The difference between the PCNA index in larval foregut (FG) sections of the negative control compared with the 0.2% MOS equals 2.3625 (95% CI [-5.0890; 9.8140]) and does not differ significantly from zero (p-value = 0.650), the negative control compared with the 0.4% MOS equals 2.3830 (95% CI [-4.8158; 9.5818]) and does not diverge significantly from zero (p-value = 0.627). The difference between the PCNA index in larval midgut (MG) sections of the negative control compared with the 0.2% MOS equals 1.2267 (95% CI [-11.0227; 13.4761]), the negative control compared with the 0.4% MOS equals 5.4660 (95% CI [-7.0859; 18.0179]) and both do not differ significantly from zero (p-value = 0.959 and 0.482, respectively).

The difference between the TUNEL index in larval FG sections of the negative control compared with the 0.2% MOS equals 0.8200 (95% CI [-0.9984; 2.6384]) and does not differ significantly from zero (p-value = 0.438), the negative control compared with the 0.4% MOS equals -0.0860 (95% CI [-1.8427; 1.6707]) and does also not differ significantly from zero (p-value = 0.989). The difference between the TUNEL index in larval MG sections of the negative control compared with the 0.2% MOS equals 1.5667 (95% CI [-2.7766; 5.9100]), the negative control compared with the 0.4% MOS equals -1.2400 (95% CI [-5.6906; 3.2106]) and both do not differ significantly from zero (p-value = 0.731 and 0.598, respectively).

7.5 DISCUSSION

Following administration of alginates, increased fish growth and non-specific immunity were observed in adults of various freshwater species (Fujiki & Yano, 1997; Peddie et al., 2002) as well as in seabass (*Dicentrarchus labrax*) (Bagni et al., 2005). With regard to flatfish larvae, increased protein turnover, growth and survival were noted in Atlantic halibut (*Hippoglossus hippoglossus*) and turbot (*Scophthalmus maximus*) (Vadstein et al., 1993; Conceicao et al., 2001; Skjermo & Bergh, 2004). In contrast, in the present study on Dover sole larvae, a significant decrease in standard body length was observed following 0.4% AA supplementation compared with the non-supplemented group. Furthermore, a significant decrease in length at 21 DAH was encountered in the larvae supplemented with 0.4% AA followed by pathogen challenge compared with the non-supplemented larvae challenged with *V. anguillarum* WT (positive control). When reading these data, it is important to state that the sampling sizes were very limited. Indeed, due to the high mortality rates, only three and six larvae were measured in the 0.2% and 0.4% AA supplemented groups, respectively. Therefore, caution is needed when interpreting the latter results. Improved performance of alginate supplemented animals following pathogen challenge was observed in adult (Fujiki et al., 1994; Fujiki & Yano, 1997; Cheng et al., 2007) and juvenile fish (Skjermo et al., 1995; Skjermo & Bergh, 2004; Vollstad et al., 2006; Cui et al., 2012). To our knowledge, this study is the first to evaluate survival after challenge with a pathogen following prebiotic administration in fish larvae. Including such a challenge makes it possible to evaluate not only the direct impact of prebiotic supplementation on larval growth and survival, but also its indirect impact following the infliction of a stressor (in this case pathogen challenge). No increased survival following challenge was observed in the supplemented groups. In contrast, a significantly decreased survival was noted in the larvae supplemented with 0.2% AA following pathogen challenge compared with the non-supplemented larvae challenged with the pathogen (positive control). No significant difference was noticed between the group with the 0.4% AA supplementation and the positive control following challenge although a negative trend was detected ($p = 0.0252$). In contrast, no significant differences in mortality between supplemented larvae and the negative control were observed. These results might suggest that diet supplementation with AA does not seem to have a direct negative impact on larval growth and survival but may indicate a possible indirect negative impact, underscoring the importance of including both direct and indirect parameters when evaluating the harmlessness of new compounds.

Alginic acid sodium salt is used as the main component of alginate microparticles (Yaacob et al., 2017), which may be applied for delivery of bacteria (Gbassi et al., 2009), drugs or even oral vaccination in fish (Joosten et al., 1997; Romalde et al., 2004). Furthermore, incorporation of immunostimulants and antigens into micro- and nanoparticles is suggested to be a new tool in fish treatment. This method

allows controlled delivery as well as improved targeting and stimulation of the immune system (Ji et al., 2015). In seabass larvae, protection against *V. anguillarum* by oral administration of encapsulated immunostimulants (ferritin-H and DnaK) in alginate microparticles was evaluated (Yaacob, 2017; Yaacob et al., 2018). Altered gene expression levels were observed although no increased survival was noted. Furthermore, empty calcium alginate microparticles by themselves did not induce a negative impact on the survival of seabass larvae (Yaacob et al., 2017) nor resulted in an increased mortality following *Vibrio* challenge (Yaacob et al., 2018). However, the larvae were only monitored for five days post treatment and were not fed during the experiments. In this study, a possible adverse effect of AA on Dover sole larvae was observed although again the small sampling size needs to be underscored. This might indicate that caution may be needed when using alginate particles as an immunostimulant or drug carrier in Dover sole larvae. Next to a possible effect on the larvae, the apparent negative impact on larval length and survival following challenge may also be rooted in a possible interaction between AA and the *Artemia*. Further research hence is imperative to determine the harmlessness of components based on alginic acid sodium salt to fish larvae.

Dietary MOS supplementation was shown to improve gut morphology, resulting in an increased food conversion and therefore improved growth in adult and juvenile fish (e.g. Staykov et al., 2007; Yilmaz et al., 2007; Dimitroglou et al., 2010). In contrast, no increased growth or survival was observed in larvae of white seabream (Dimitroglou et al., 2010) and cobia (Salze et al., 2008) nor in the current study on Dover sole larvae supplemented with MOS. In adult fish, increased mucosal folding and villi length were observed (Yilmaz et al., 2007; Dimitroglou et al., 2009; 2011). Intestinal folding is not fully completed in Dover sole larvae (Veggetti et al., 1999) and this parameter was therefore not included in the present study. Furthermore, to evaluate mucosal folding in the intestine, the fish should be fastened, ensuring an empty intestine. Small larvae do not tolerate starvation well in comparison with juvenile or adult fish, impeding the use of these measurements in larval research.

As stated before, improved resistance against pathogens following MOS supplementation was reported for several marine fish species (seabass (Torrecillas et al., 2007; 2011; 2012), Nile tilapia (*Oreochromis niloticus*) (El-Boshy et al., 2010), Senegalese sole (*Solea senegalensis*) (Dimitroglou et al., 2010), cod (*Gadus morhua*) (Lokesh et al., 2012)). To our knowledge, hitherto, no studies investigated the protective potential of prebiotic supplementation following pathogen challenge in fish larvae, highlighting the importance of this study. The mode of action of MOS to reduce pathogen susceptibility in fish is still not well understood. Many infections are initiated by bacterial colonization of the gut mucosa. To become pathogenic, bacteria need to attach to the mucus and multiply quickly before being translocated outside the intestine, followed by adherence to the cell wall of enterocytes. This adherence is based on their interaction with carbohydrates on the cell surface (Bavington & Page,

2005). In broilers, dietary MOS was reported to bind with pathogens, preventing them to adhere to the cell surface of the enterocytes and consequently colonizing the gut (Spring et al., 2000). Without attachment, pathogens pass through the intestine and are excreted, reducing the incidence and severity of infections (Spring et al., 2000; Sonone et al., 2011). Pathogen binding was also suggested to be the underlying mechanism for increased survival in juvenile fish following *V. anguillarum* and *V. alginolyticus* challenge (Torrecillas et al., 2007; 2011a; 2011b) and adults of the closely related Senegalese sole infected by *Photobacterium damselae* subsp. *piscicida* (Dimitroglou et al., 2011). However, in the present study on Dover sole larvae, no protection following *V. anguillarum* challenge was observed.

It is important to highlight that the high mortality in the negative control group in both experiments, might suggest a low egg and/or larval quality. Indeed, variable egg and larval quality between batches was observed in many fish species, including Dover sole (Lund, 2007; Bolle et al., 2012). The low survival in comparison to previous studies (De Swaef et al., 2017; 2018) may also be accounted for by the omission of disinfection of the Dover sole eggs prior to incubation in glass bottles and multiwell plates due to early hatching. A higher bacterial load may indeed have impacted the larval quality, resulting in an overall lower survival. Furthermore, suboptimal larval quality may exacerbate negative effects on survival when adding additional stress factors such as a pathogen challenge. This may (partially) explain the very low survival rates of challenged groups compared with earlier experiments including this infection model (De Swaef et al., 2018).

Larvae were housed individually in a multiwell housing system previously developed for Dover sole (De Swaef et al., 2017). Multiwell plates are frequently used in pathogenesis studies (Bergh et al., 1997; Sandlund & Bergh, 2008; Sandlund et al., 2010; Rønneseth et al., 2017) and studies involving probiotics (Makridis et al., 2000; 2001; Planas et al., 2005; 2006; Schaeck et al., 2016). To our knowledge, hitherto, no studies investigating the impact of prebiotics on fish (larvae) were performed using such an individual housing system. However, the advantages of a multiwell housing system previously described for probiotic research also apply here (Schaeck et al., 2016; De Swaef et al., 2017; 2018). Indeed, housing the larvae individually has the advantage that mortality of one individual has no impact on the adjacent one. Furthermore, the experimental set-up provides a high number of individual measurements. Next to survival and growth, the well plate also makes it possible to evaluate additional parameters such as behaviour or stress response in prebiotic research.

Both prebiotic candidates were administered to the larvae via biocapsulation by supplementing the rearing water of the newly hatched *Artemia* nauplii with MOS or AA at the described concentrations. The solubility of the prebiotics in water appeared limited. Mannanoligosaccharide seemed completely

dissolved in the water but strong mixing and aeration was needed. To evaluate the effect of AA, alginic acid sodium salt was used in order to ensure a high solubility of the otherwise undissolvable AA. However, even with high mixing and aeration, the product was not completely dissolved, generating small gelatinous beads at the bottom of the beakers. Therefore, the final concentration of AA to which the *Artemia* nauppli were exposed, is expected to be lower than the actual concentration added to the rearing water. Since the *Artemia* were rinsed before feeding to the larvae, the gelatinous beads were not distributed to the larvae in the multiwell plates and therefore can not explain the lower larval survival.

Focusing on fish, histological analysis of the digestive system is considered a good indicator of the health status of the gut. The intestinal epithelium undergoes rapid cell turnover and changes in both cell proliferation, differentiation and apoptosis may impair the normal cell renewal and indicate alterations in larval health (Yang et al., 2009). Immunohistochemical analyses were only performed on Dover sole larvae supplemented with MOS. Indeed, since an apparent negative impact of AA supplementation was observed in the larvae, no additional studies investigating a possible positive impact were performed on these animals. In contrast, although no increased survival or growth and no protection following pathogen challenge was observed for the MOS supplemented fish larvae, no adverse effects were noted either. Therefore, it seemed important to verify possible (positive) changes in the cell kinetics of the gastrointestinal tract which might have been present without resulting in increased survival or growth at 10 DAH. To evaluate cell proliferation, an immunohistochemical staining of PCNA was performed. PCNA is a highly conserved protein involved in DNA synthesis (Ortego et al., 1994). This technique is also repeatedly used in toxicity tests including different fish species since changes in the rate of normal cell proliferation may be an early indicator of abnormality (Ortego et al., 1995; Ferrando et al., 2005; Sanden & Olsvik, 2009). In adult fish, PCNA positive cell nuclei in the intestines are mainly located at the base of the villi (Ferrando et al., 2005). However, in fish larvae the degree of cell proliferation is much higher, as can be expected in developing organisms. Indeed, in this study, Dover sole larvae at 10 DAH exhibited a PCNA index exceeding 85% which corresponds with a former study on Dover sole larval development (Vegetti et al., 1999). In contrast, gnotobiotic seabass larvae of 10 DAH displayed PCNA indices between 20 and 35% (Schaeck et al., 2017). Indeed, reduced epithelial proliferation (although based on genomic data) was also detected in gnotobiotic zebrafish larvae (Rawls et al., 2007) and may therefore be linked to the gnotobiotic condition of the animals rather than the fish species. No significant differences between the PCNA index of the prebiotic supplemented and control larvae were observed. To evaluate the apoptosis in enterocytes, a immunohistochemical TUNEL assay was established but no significant differences between treatments were noted. In general, the apoptotic index remained very low which was also in accordance with a

study on larvae of zebrafish (Ng et al., 2005) and seabass (Schaeck et al., 2017) at early development. Hitherto, studies on the normal proliferation and apoptotic ratios in fish larvae are limited. Furthermore, to our knowledge, no previous experiments evaluating pro- or prebiotic administration on fish larvae included PCNA and apoptosis indices to evaluate the cell kinetics in the gut, highlighting the completeness of this study.

Immunohistochemical analyses were performed on both foregut and midgut sections of Dover sole larvae. However, determination of the appropriate intestinal section is difficult in flatfish due to the specific orientation of the organs in the small and cranially located abdominal cavity. Whereas in larvae of most round fish the intestine has the appearance of a straight tube (Govoni et al., 1986), organogenesis is complex in flatfish. Indeed, in Dover sole the intestine is already slightly curved at hatching and by 10 DAH, the anus migrates towards the head, resulting in a lengthening of the gut (Boulhic & Gabaudan, 1992). Following this, large changes in the position of the intestine occur. To our knowledge, in the published literature, no fully detailed description of the intestinal displacement is available. Following 3D reconstruction of the gut of Dover sole larvae at different stages between 6 to 26 DAH, it was noted that at 10 DAH, the intestinal tube may be considered a long convoluted tube or a looping, depending on the individual and its developmental stage (data not published). This looping of the intestinal tract of flatfish was also observed following 3D reconstruction in Atlantic halibut larvae (Gomes et al., 2014). Therefore, the exact position of the transversal section was not identified during the histological examination. At 10 DAH, the stomach is not delineated. However, the oesophagus may be recognized due to the presence of a high number of goblet cells (Boulhic & Gabaudan, 1992; Veggetti et al., 1999). Histological sections were collected 10 µm caudally from the oesophagus and this area was defined as foregut. Delineation of the boundary between oesophagus and foregut is of major importance for the apoptotic index, since large numbers of apoptotic cells were observed in the oesophagus sections but not in the foregut. Consequently, several foregut sections were not included in the results as goblet cells appeared abundantly present after staining and therefore indicated that part of the oesophagus was present. In almost all larvae, a second transverse section of the intestine was present on the same slide and this section was defined as midgut, although the exact position in the intestinal tract was not determined. However, due to the relatively cranial position of the oesophagus, the possibility of sectioning the rectal region at this location was limited. Differential effects of prebiotic treatment between cranial and caudal intestine in gilthead seabream were reported (Dimitroglou et al., 2010), justifying the inclusion of two intestinal regions in this trial. However, in small fish larvae, differentiation of the intestine is still in full progress and is therefore much less delineated than in adult fish.

In conclusion, this study investigated for the first time the effect of prebiotic supplementation in Dover sole larvae, hereby including not only direct but also indirect parameters by pathogen challenge with *Vibrio anguillarum*. Although increased fish health and performance were reported for other fish species, no increased larval growth nor shifted proliferation and apoptosis rate of the intestinal epithelium were observed in Dover sole larvae following 0.2% or 0.4% MOS supplementation. In contrast, a decreased standard body length was noted following 0.4% alginic acid supplementation but no differences in cell kinetics were detected. No increased survival was observed following administration of both compounds. However, a decreased survival at 21 DAH of the larvae supplemented with 0.2% alginic acid followed by pathogen challenge compared with the challenged non-supplemented larvae was observed, hereby highlighting the importance to include both direct as well as indirect parameters to evaluate the harmlessness of new compounds.

7.6 ACKNOWLEDGEMENTS

We acknowledge Christian Puttevils, Delphine Ameye and Joachim Christiaens for the outstanding technical assistance and help during the experiments. Furthermore, the authors would like to thank Alain Le Breton for kindly providing the *V. anguillarum* WT isolate. Stichting Zeeschelp (the Netherlands) are gratefully acknowledged for providing the *S. solea* eggs.

Table 7.1 - PCNA and TUNEL index of the foregut and midgut sections for control larvae and larvae supplemented with 0.2% or 0.4% MOS. For both PCNA and TUNEL, the mean index is given for the foregut and midgut section per larvae. Furthermore, the number of sections analysed to calculate the mean index was mentioned between brackets. - = no sections available

		PCNA index		TUNEL index	
Treatment	Larva	Foregut	Midgut	Foregut	Midgut
		Mean index (%) (n= number of sections)	Mean index (%) (n= number of sections)	Mean index (%) (n= number of sections)	Mean index (%) (n= number of sections)
Control	1	94.75 (n=4)	85.80 (n=5)	0.17 (n=6)	0.00 (n=6)
	2	96.33 (n=6)	98.67 (n=3)	0.17 (n=6)	0.00 (n=3)
	3	96.17 (n=6)	96.50 (n=6)	-	1.50 (n=4)
	4	-	97.33 (n=6)	-	1.20 (n=5)
	5	98.40 (n=5)	98.67 (n=4)	0.83 (n=6)	0.17 (n=6)
	6	94.33 (n=3)	97.67 (n=6)	0.75 (n=4)	0.33 (n=3)
0.2% MOS	1	97.20 (n=5)	95.40 (n=5)	1.33 (n=6)	1.80 (n=5)
	2	99.60 (n=5)	93.60 (n=5)	3.00 (n=4)	6.50 (n=4)
	3	96.83 (n=6)	96.00 (n=6)	1.20 (n=5)	5.20 (n=5)
	4	96.83 (n=6)	95.67 (n=6)	0.40 (n=5)	0.00 (n=4)
0.4% MOS	1	98.83 (n=3)	95.00 (n=5)	1.00 (n=5)	0.00 (n=3)
	2	97.80 (n=3)	99.00 (n=4)	1.60 (n=5)	4.20 (n=5)

Table 7.2 - Overview of the survival (%) and mean standard body length (µm) of Dover sole larvae at 21 DAH. Larvae were supplemented with alginic acid or mannanoligosaccharide from 4 to 9 DAH at two concentrations (0.2% or 0.4%), whether or not followed by challenge with V. anguillarum *WT at 10 DAH. A negative control group consisted of larvae with no bacterial cells added. Survival and standard body length were compared with the supplemented groups without challenge. A positive control group comprising larvae challenged with* V. anguillarum *at 10 DAH, without probiotic supplementation was compared with the groups supplemented prior to pathogen challenge. Different letters (a, b, c & d) indicate statistically significant differences at p<0.025. n= number of larvae included to calculated the mean standard body length.*

	Treatment group	**Survival (%)**	**Mean standard body length (µm)(n=number of larvae)**
Experiment 1	Negative control	49^{a}	7105.47^{a} (n=20)
	0.2% algic acid	47^{a}	7370.00^{a} (n=3)
	0.4% alginic acid	42^{a}	6273.61^{b} (n=6)
	V. anguillarum WT	36^{b}	6670.98^{c} (n=20)
	0.2% alginic acid *+ V. anguillarum* WT	16^{c}	6087.32^{c} (n=4)
	0.4% alginic acid *+ V. anguillarum* WT	21^{b}	5892.41^{d} (n=5)
Experiment 2 (feed)	Negative control	53^{a}	6994.99^{a} (n=20)
	0.2% mannanoligosaccharide	66^{a}	7370.00^{a} (n=20)
	0.4% mannanoligosaccharide	53^{a}	6273.61^{a} (n=20)
	V. anguillarum WT	15^{b}	6293.77^{a} (n=20)
	0.2% mannanoligosaccharide *+ V. anguillarum* WT	17^{b}	6931.99^{a} (n=20)
	0.4% mannanoligosaccharide *+ V. anguillarum* WT	16^{b}	6767.88^{a} (n=20)

7.7 REFERENCES

Akbari, M., Heidarieh, M., Mirvaghefi, A., Farahmand, H., Sheikhzadeh, N. & Hajivar, E.N. (2015) Effect of dietary Ergosan and Hilyses on growth performance, haematological variables and immune response in rainbow trout (*Oncorhynchus mykiss*). *Iranian Journal of Aquatic Animal Health,* **1**, 1-6.

Austin, B. & Austin, D.A. (2012) *Bacterial Fish Pathogens: disease of farmed and wild fish*. Fifth edition. Springer Dordrecht

Bagni, M., Romano, N., Finoia, M.G., Abelli, L., Scapigliati, G., Tiscar, P.G., Sarti, M. & Marino, G. (2005) Short- and long-term effects of a dietary yeast beta-glucan (Macrogard) and alginic acid (Ergosan) preparation on immune response in sea bass (*Dicentrarchus labrax*). *Fish & Shellfish Immunology,* **18,** 311-325.

Bavington, C. & Page, C. (2005) Stopping bacterial adhesion: A novel approach to treating infections. *Respiration,* **72,** 335-344.

Bergh, O., Hjeltnes, B. & Skiftesvik, A.B. (1997) Experimental infection of turbot *Scophthalmus maximus* and halibut *Hippoglossus hippoglossus* yolk sac larvae with *Aeromonas salmonicida* subsp *salmonicida*. *Diseases of Aquatic Organisms,* **29,** 13-20.

Bjørndal, T., Guillen, J. & Imsland, A. (2016) The potential of aquaculture sole production in Europe: Production costs and markets. *Aquaculture Economics & Management,* **20,** 109-129.

Bjørndal, T., Guillen, J., Imsland, A. & Øiestad, V. (2015) Sole: production and market. GLOBEFISH Research Programme, Rome, FAO, 118, 1-72.

Bolle, L.J., De Jong, C.a.F., Bierman, S.M., Van Beek, P.J.G., Van Keeken, O.A., Wessels, P.W., Van Damme, C.J.G., Winter, H.V., De Haan, D. & Dekeling, R.P.A. (2012) Common Sole Larvae Survive High Levels of Pile-Driving Sound in Controlled Exposure Experiments. *Plos One,* **7**.

Boulhic, M. & Gabaudan, J. (1992) Histological Study of the Organogenesis of the Digestive-System and Swim Bladder of the Dover Sole*, Solea Solea* (Linnaeus 1758). *Aquaculture,* **102,** 373-396.

Bricknell, I. & Dalmo, R.A. (2005) The use of immunostimulants in fish larval aquaculture. *Fish & Shellfish Immunology,* **19,** 457-472.

Burridge, L., Weis, J.S., Cabello, F., Pizarro, J. & Bostick, K. (2010) Chemical use in salmon aquaculture: A review of current practices and possible environmental effects. *Aquaculture,* **306,** 7-23.

Caipang, C.M.A., Lazado, C.C., Berg, I., Brinchmann, M.F. & Kiron, V. (2011) Influence of alginic acid and fucoidan on the immune responses of head kidney leukocytes in cod. *Fish Physiology and Biochemistry,* **37,** 603-612.

Carbone, D. & Faggio, C. (2016) Importance of prebiotics in aquaculture as immunostimulants. Effects on immune system of *Sparus aurata* and *Dicentrarchus labrax*. *Fish & Shellfish Immunology,* **54,** 172-178.

Cheng, A.C., Tu, C.W., Chen, Y.Y., Nan, F.H. & Chen, J.C. (2007) The immunostimulatory effects of sodium alginate and iota-carrageenan on orange-spotted grouper *Epinephelus coicoides* and its resistance against *Vibrio alginolyticus*. *Fish & Shellfish Immunology,* **22,** 197-205.

Conceicao, L.E.C., Skjermo, J., Skjak-Braek, G. & Verreth, J.a.J. (2001) Effect of an immunostimulating alginate on protein turnover of turbot (*Scophthalmus maximus* L.) larvae. *Fish Physiology and Biochemistry,* **24,** 207-212.

Cui, L., Xu, W., Ai, Q., Wang, D. & Mai, K. (2012) Effects of dietary chitosan oligosaccharide complex with rare earth on growth performance and innate immune response of turbot, *Scophthalmus maximus* L. *Aquaculture Research,* **44**, 683–690.

De Swaef, E., Demeestere, K., Boon, N., Van Den Broeck, W., Haesebrouck, F. & Decostere, A. (2017) Development of a reliable experimental set-up for Dover sole larvae *Solea solea* L. and exploring the possibility of implementing this housing system in a gnotobiotic model. *Research in Veterinary Science,* **115,** 418-424.

De Swaef, E., Vercauteren, M., Duchateau, L., Haesebrouck, F. & Decostere, A. (2018) Experimental infection model CrossMark for vibriosis in Dover sole (*Solea solea*) larvae as an aid in studying its pathogenesis and alternative treatments. *Veterinary Research,* **49**.

Dimitroglou, A., Merrifield, D.L., Carnevali, O., Picchietti, S., Avella, M., Daniels, C., Guroy, D. & Davies, S.J. (2011) Microbial manipulations to improve fish health and production - A Mediterranean perspective. *Fish & Shellfish Immunology,* **30,** 1-16.

Dimitroglou, A., Merrifield, D.L., Moate, R., Davies, S.J., Spring, P., Sweetman, J. & Bradley, G. (2009) Dietary mannan oligosaccharide supplementation modulates intestinal microbial ecology and improves gut morphology of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Animal Science,* **87,** 3226-3234.

Dimitroglou, A., Merrifield, D.L., Spring, P., Sweetman, J., Moate, R. & Davies, S.J. (2010) Effects of mannan oligosaccharide (MOS) supplementation on growth performance, feed utilisation, intestinal histology and gut microbiota of gilthead sea bream (*Sparus aurata*). *Aquaculture,* **300,** 182-188.

El-Boshy, M.E., El-Ashram, A.M., Abdelhamid, F.M. & Gadalla, H.A. (2010) Immunomodulatory effect of dietary Saccharomyces cerevisiae, beta-glucan and laminaran in mercuric chloride treated Nile tilapia (*Oreochromis niloticus*) and experimentally infected with *Aeromonas hydrophila*. *Fish & Shellfish Immunology,* **28,** 802-808.

European Union DIVERSIFY project (2016) http://www.diversifyfish.eu/ (accessed: 26/07/2018)

FAO (2018) Food and Agriculture Organization of the United Nations, www.fao.org. (Accessed on 15/12/2018)

Ferrando, S., Ferrando, T., Girosi, L., Mauceri, A., Fasulo, S. & Tagliafierro, G. (2005) Apoptosis, cell proliferation and serotonin immunoreactivity in gut of *Liza aurata* from natural heavy metal polluted environments: preliminary observations. *European Journal of Histochemistry*, **49**, 331-340.

Ferraresso, S., Bonaldo, A., Parma, L., Cinotti, S., Massi, P., Bargelloni, L. & Gatta, P.P. (2013) Exploring the larval transcriptome of the common sole (*Solea solea* L.). *Bmc Genomics,* **14**.

Fujiki, K., Matsuyama, H. & Yano, T. (1994) Protective Effect of Sodium Alginates against Bacterial-Infection in Common Carp, *Cyprinus Carpio* L. *Journal of Fish Diseases,* **17,** 349-355.

Fujiki, K. & Yano, T. (1997) Effects of sodium alginate on the non-specific defence system of the common carp (*Cyprinus carpio* L). *Fish & Shellfish Immunology,* **7,** 417-427.

Gbassi, G.K., Vandamme, T., Ennahar, S. & Marchioni, E. (2009) Microencapsulation of *Lactobacillus plantarum* spp in an alginate matrix coated with whey proteins. *International Journal of Food Microbiology,* **129,** 103-105.

Gioacchini, G., Smith, P. & Carnevali, O. (2008) Effects of Ergosan on the expression of cytokine genes in the liver of juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to enteric red mouth vaccine. *Veterinary Immunology and Immunopathology,* **123,** 215-222.

Gomes, A.S., Kamisaka, Y., Harboe, T., Power, D.M. & Rønnestad, I. (2014) Functional modifications associated with gastrointestinal tract organogenesis during metamorphosis in Atlantic halibut (*Hippoglossus hippoglossus*). *Bmc Developmental Biology,* **14**.

Govoni, J.J., Boehlert, G.W. & Watanabe, Y. (1986) The Physiology of Digestion in Fish Larvae. *Environmental Biology of Fishes,* **16,** 59-77.

Hickey, M.E. & Lee, J.L. (2018) A comprehensive review of *Vibrio (Listonella) anguillarum*: ecology, pathology and prevention. *Reviews in Aquaculture,* **10,** 585-610.

Hoseinifar, S.H., Esteban, M.A., Cuesta, A. & Sun, Y.Z. (2015) Prebiotics and Fish Immune Response: A Review of Current Knowledge and Future Perspectives. *Reviews in Fisheries Science & Aquaculture,* **23,** 315-328.

Ji, J., Torrealba, D., Ruyra, A. & Roher, N. (2015) Nanodelivery Systems as New Tools for Immunostimulant or Vaccine Administration: Targeting the Fish Immune System. *Biology*, **4,** 665-696.

Joosten, P.H.M., Tiemersma, E., Threels, A., Caumartindhieux, C. & Rombout, J.H.W.M. (1997) Oral vaccination of fish against *Vibrio anguillarum* using alginate microparticles. *Fish & Shellfish Immunology,* **7,** 471-485.

Kakuta, I. (2004) Enhancement of the Biodefense Activity and Improvement of Physiological Condition of Fish by Oral Administration of Algae Fucoidan. *Suisanzoshoku*, **52**, 413-420.

Lane, A., Hough, C. & Bostock, J. (2014) The long-term economic and ecologic impact of larger sustainable aquaculture. Study for the European Parliament, Directorate General for Internal Policies, Policy Department B, Structural and Cohesion Policies—Fisheries.

Lokesh, J., Fernandes, J.M.O., Korsnes, K., Bergh, O., Brinchmann, M.F. & Kiron, V. (2012) Transcriptional regulation of cytokines in the intestine of Atlantic cod fed yeast derived mannan oligosaccharide or beta-Glucan and challenged with *Vibrio anguillarum*. *Fish & Shellfish Immunology,* **33,** 626-631.

Lund, I. (2007) The impact of dietary fatty acids in common sole larval (*Solea solea* L.) nutrition. PhD dissertation, Department of Aquaculture, Danish Technical University, Danmark.

Makridis, P., Bergh, Ø., Skjermo, J. & Vadstein, O. (2001) Addition of bacteria bioencapsulated in *Artemia* metanauplii to a rearing system for halibut larvae. *Aquaculture International,* **9,** 225-235.

Makridis, P., Jon Fjellheim, A., Skjermo, J. & Vadstein, O. (2000) Colonization of the gut in first feeding turbot by bacterial strains added to the water or bioencapsulated in rotifers. *Aquaculture International,* **8,** 367-380.

Morales-Lange, B., Bethke, J., Schmitt, P. & Mercado, L. (2015) Phenotypical parameters as a tool to evaluate the immunostimulatory effects of laminarin in *Oncorhynchus mykiss*. *Aquaculture Research,* **46,** 2707-2715.

Ng, A.N.Y., De Jong-Curtain, T.A., Mawdsley, D.J., White, S.J., Shin, J., Appel, B., Dong, P.D.S., Stainier, D.Y.R. & Heath, J.K. (2005) Formation of the digestive system in zebrafish: III. Intestinal epithelium morphogenesis. *Developmental Biology,* **286,** 114-135.

Ortego, L.S., Hawkins, W.E., Walker, W.W., Krol, R.M. & Benson, W.H. (1994) Detection of Proliferating Cell Nuclear Antigen in Tissues of 3 Small Fish Species. *Biotechnic & Histochemistry,* **69,** 317-323.

Ortego, L.S., Hawkins, W.E., Walker, W.W., Krol, R.M. & Benson, W.H. (1995) Immunohistochemical Detection of Proliferating Cell Nuclear Antigen (PCNA) in Tissues of Aquatic Animals Utilized in Toxicity Bioassays. *Marine Environmental Research,* **39,** 271-273.

Paolini, A., Magi, G.E., Gennari, L., Egidi, C., Torresi, M., Vallerani, M., Berti, M., Pavone, A. & Giorgetti, G. (2010). Severe mortality in common sole (*Solea solea*) at post-weaning stage following environmental stress. *Bulletin of the European Association of Fish Pathologists,* **30**, 160-169.

Palazzi, R., Richard, J., Bozzato, G. & Zanella, L. (2006) Larval and juvenile rearing of common sole (*Solea solea* L.) in the Northern Adriatic (Italy). *Aquaculture,* **255,** 495-506.

Peddie, S., Zou, J. & Secombes, C.J. (2002) Immunostimulation in the rainbow trout (*Oncorhynchus mykiss*) following intraperitoneal administration of Ergosan. *Veterinary Immunology and Immunopathology,* **86,** 101-113.

Planas, M., Perez-Lorenzo, M., Hjelm, M., Gram, L., Fiksdal, I.U., Bergh, O. & Pintado, J. (2006) Probiotic effect *in vivo* of *Roseobacter* strain 27-4 against *Vibrio (Listonella) anguillarum* infections in turbot (*Scophthalmus maximus* L.) larvae. *Aquaculture,* **255,** 323-333.

Planas, M., Perez-Lorenzo, M., Vazquez, J.A. & Pintado, J. (2005) A model for experimental infections with *Vibrio (Listonella) anguillarum* in first feeding turbot (*Scophthalmus maximus* L.) larvae under hatchery conditions. *Aquaculture,* **250,** 232-243.

Rawls, J.F., Mahowald, M.A., Goodman, A.L., Trent, C.M. & Gordon, J.I. (2007) In vivo imaging and genetic analysis link bacterial motility and symbiosis in the zebrafish gut. *Proceedings of the National Academy of Sciences of the United States of America,* **104,** 7622-7627.

Ringø, E., Olsen, R.E., Gifstad, T.O., Dalmo, R.A., Amlund, H., Hemre, G.I. & Bakke, A.M. (2010) Prebiotics in aquaculture: a review. *Aquaculture Nutrition,* **16,** 117-136.

Ringø, E., Olsen, R.E., Jensen, I., Romero, J. & Lauzon, H.L. (2014) Application of vaccines and dietary supplements in aquaculture: possibilities and challenges. *Reviews in Fish Biology and Fisheries,* **24,** 1005-1032.

Ringø, E. & Song, S.K. (2016) Application of dietary supplements (synbiotics and probiotics in combination with plant products and -glucans) in aquaculture. *Aquaculture Nutrition,* **22,** 4-24.

Rodgers, P.E., Read, A. & Meun, G. (2005) Benefits of Responsible Fishing: The impact of an innovative trial of voluntary restraint. Report for the EU Directorate-General for Fisheries, Contract No. 2003/C 115/08-17, CFER, Lincoln.

Rodriguez-Estrada, U., Satoh, S., Haga, Y., Fushimi, H. & Sweetman, J. (2009) Effects of Single and Combined Supplementation of *Enterococcus faecalis*, Mannan Oligosaccharide and Polyhydroxybutyrate Acid on Growth Performance and Immune Response of Rainbow Trout *Oncorhynchus mykiss*. *Aquaculture Science*, **57**, 609-617.

Romalde, J.L., Luzardo-Alvarez, A., Ravelo, C., Toranzo, A.E. & Blanco-Wendez, J. (2004) Oral immunization using alginate microparticles as a useful strategy for booster vaccination against fish lactoccocosis. *Aquaculture,* **236,** 119-129.

Romero, J., Feijoo, C.G. & Navarrete, P. (2012) Antibiotics in Aquaculture – Use, Abuse and Alternatives. In: Carvalho ED, David GS, Silva RJ (eds) *Health and Environment in Aquaculture*. InTech, Rijeka, 159-198.

Rønneseth, A., Castillo, D., D'alvise, P., Tonnesen, O., Haugland, G., Grotkjaer, T., Engell-Sorensen, K., Norremark, L., Bergh, O., Wergeland, H.I. & Gram, L. (2017) Comparative assessment of *Vibrio* virulence in marine fish larvae. *Journal of Fish Diseases,* **40,** 1373-1385.

Salze, G., Mclean, E., Schwarz, M.H. & Craig, S.R. (2008) Dietary mannan oligosaccharide enhances salinity tolerance and gut development of larval cobia. *Aquaculture,* **274,** 148-152.

Sanden, M. & Olsvik, P.A. (2009) Intestinal cellular localization of PCNA protein and CYP1A mRNA in Atlantic salmon *Salmo salar* L. exposed to a model toxicant. *BMC Physiology*, **9**.

Sandlund, N. & Bergh, O. (2008) Screening and characterisation of potentially pathogenic bacteria associated with Atlantic cod *Gadus morhua* larvae: bath challenge trials using a multidish system. *Diseases of Aquatic Organisms,* **81,** 203-217.

Sandlund, N., Rodseth, O.M., Knappskog, D.H., Fiksdal, I.U. & Bergh, O. (2010) Comparative susceptibility of turbot, halibut, and cod yolk-sac larvae to challenge with *Vibrio* spp. *Diseases of Aquatic Organisms,* **89,** 29-37.

Schaeck, M., Duchateau, L., Van Den Broeck, W., Van Trappen, S., De Vos, P., Coulombet, C., Boon, N., Haesebrouck, F. & Decostere, A. (2016) *Vibrio lentus* protects gnotobiotic sea bass (*Dicentrarchus labrax* L.) larvae against challenge with *Vibrio harveyi. Veterinary Microbiology,* **185,** 41-48.

Schaeck, M., Reyes-Lopez, F.E., Vallejos-Vidal, E., Van Cleemput, J., Duchateau, L., Van Den Broeck, W., Tort, L. & Decostere, A. (2017) Cellular and transcriptomic response to treatment with the probiotic candidate *Vibrio lentus* in gnotobiotic sea bass (*Dicentrarchus labrax*) larvae. *Fish & Shellfish Immunology,* **63,** 147-156.

Skjermo, J., Bakke, I., Dahle, S.W. & Vadstein, O. (2015) Probiotic strains introduced through live feed and rearing water have low colonizing success in developing Atlantic cod larvae. *Aquaculture,* **438,** 17-23.

Skjermo, J. & Bergh, O. (2004) High-M alginate immunostimulation of Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae using *Artemia* for delivery, increases resistance against vibriosis. *Aquaculture,* **238,** 107-113.

Skjermo, J., Defoort, T., Dehasque, M., Espevik, T., Olsen, Y., Skjakbraek, G., Sorgeloos, P. & Vadstein, O. (1995) Immunostimulation of Juvenile Turbot (*Scophthalmus Maximus* L) Using an Alginate with High Mannuronic Acid Content Administered Via the Live Food Organism Artemia. *Fish & Shellfish Immunology,* **5,** 531-534.

Skjermo, J. & Vadstein, O. (1999) Techniques for microbial control in the intensive rearing of marine larvae. *Aquaculture,* **177,** 333-343.

Song, S.K., Beck, B.R., Kim, D., Park, J., Kim, J., Kim, H.D. & Ringø, E. (2014) Prebiotics as immunostimulants in aquaculture: a review. *Fish & Shellfish Immunology,* **40,** 40-48.

Sonone, S., Swamy, M. & Verma, Y. (2011) Ameliorative Effect of Mannan Oligosaccharide on Pathology of Fowl Cholera in Broiler Birds. *Asian Journal of Poultry Science*, **5,** 86-93.

Spring, P., Wenk, C., Dawson, K.A. & Newman, K.E. (2000) The effects of dietary mannanoligosaccharides on cecal parameters and the concentrations of enteric bacteria in the ceca of *Salmonella*-challenged broiler chicks. *Poultry Science,* **79,** 205-211.

Staykov, Y., Spring, P., Denev, S. & Sweetman, J. (2007) Effect of a mannan oligosaccharide on the growth performance and immune status of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture International,* **15,** 153-161.

Torrecillas, S., Makol, A., Benitez-Santana, T., Caballero, M.J., Montero, D., Sweetman, J. & Izquierdo, M. (2011) Reduced gut bacterial translocation in European sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides (MOS). *Fish & Shellfish Immunology,* **30,** 674-681.

Torrecillas, S., Makol, A., Caballero, M.J., Montero, D., Dhanasiri, A.K.S., Sweetman, J. & Izquierdo, M. (2012) Effects on mortality and stress response in European sea bass, *Dicentrarchus labrax* (L.), fed

mannan oligosaccharides (MOS) after *Vibrio anguillarum* exposure. *Journal of Fish Diseases,* **35,** 591-602.

Torrecillas, S., Makol, A., Caballero, M.J., Montero, D., Robaina, L., Real, F., Sweetman, J., Tort, L. & Izquierdo, M.S. (2007) Immune stimulation and improved infection resistance in European sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides. *Fish & Shellfish Immunology,* **23,** 969-981.

Torrecillas, S., Montero, D. & Izquierdo, M. (2014) Improved health and growth of fish fed mannan oligosaccharides: Potential mode of action. *Fish & Shellfish Immunology,* **36,** 525-544.

Vadstein, O., Attramadal, K.J.K., Bakke, I., Forberg, T., Olsen, Y., Verdegem, M., Giatsis, C., Skjermo, J., Aasen, I.M., Gatesoupe, F.J., Dierckens, K., Sorgeloos, P. & Bossier, P. (2018) Managing the Microbial Community of Marine Fish Larvae: A Holistic Perspective for Larviculture. *Frontiers in Microbiology,* **9**.

Vadstein, O., Øie, G., Olsen, Y., Salvesen, I., Skjermo, J. & Skja°k-Bræk, G. (1993) A strategy to obtain microbial control during larval development of marine fish. In: Reinertsen, H., Dahle, L.A., Jørgensen, L., Tvinnereim, K. (Eds.), Fish Farming Technology. Proceedings of the First International Conference on Fish Farming Technology, 9 –12 August 1993, Trondheim, Norway. A.A. Balkema, Rotterdam, pp. 69– 75.

Vatsos, I.N. & Rebours, C. (2015) Seaweed extracts as antimicrobial agents in aquaculture. *Journal of Applied Phycology,* **27,** 2017-2035.

Veggetti, A., Rowlerson, A., Radaelli, R., Arrighi, S. & Domeneghini, C. (1999) Post-hatching development of the gut and lateral muscle in the sole. *Journal of fish Biology*, **55**, 44-65.

Vollstad, D., Bogwald, J., Gaserod, O. & Dalmo, R.A. (2006) Influence of high-M alginate on the growth and survival of Atlantic cod (*Gadus morhua* L.) and spotted wolffish (*Anarhichas minor* Olafsen) fry. *Fish & Shellfish Immunology,* **20,** 548-561.

Yaacob, E.N. (2017) Evaluation of candidate immunostimulatory compounds in gnotobiotic European sea bass (*Dicentrarchus labrax* L.) larvae, PhD dissertation, Faculty of Bioscience Engineering, University of Ghent, Belgium.

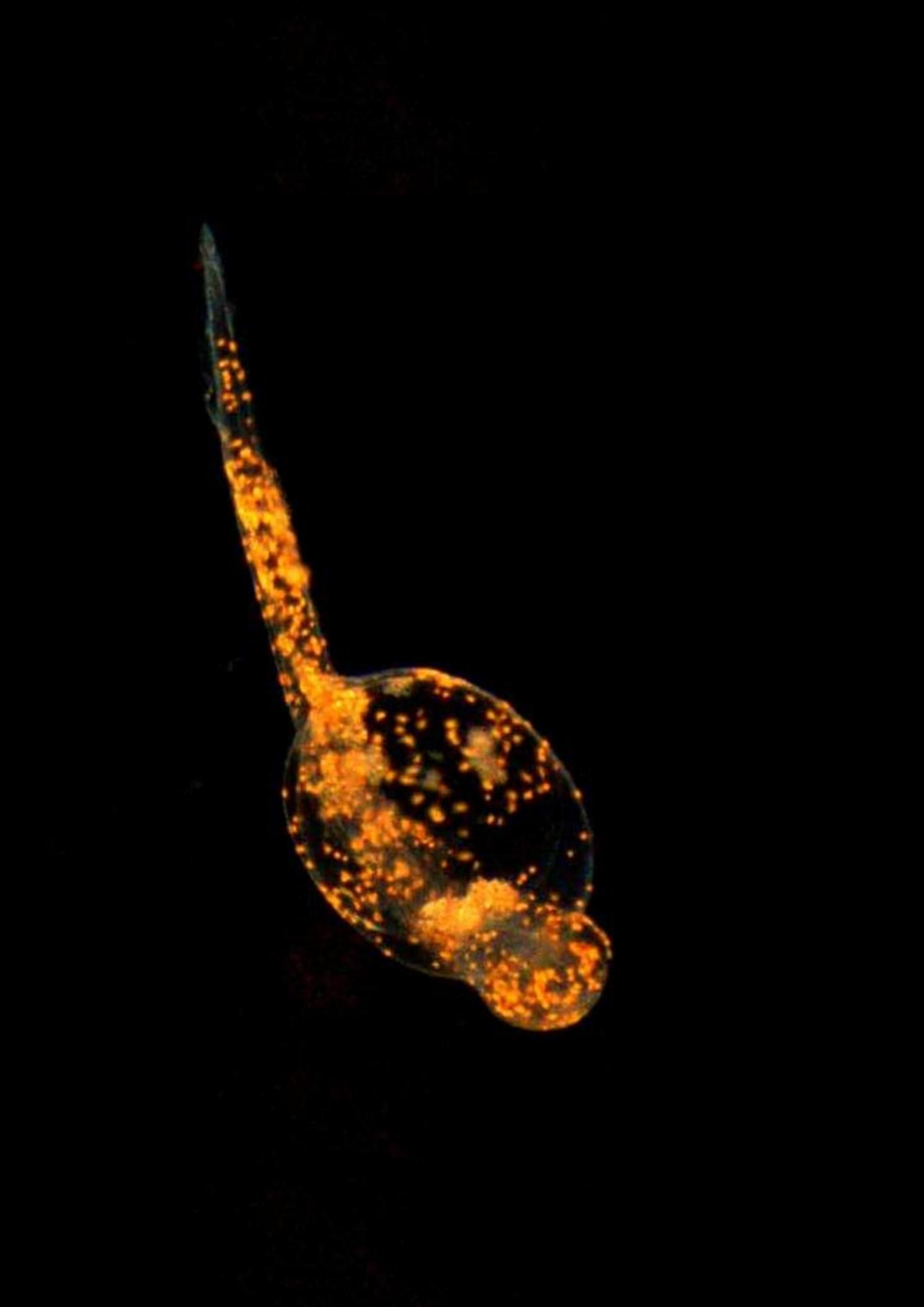
Yaacob, E.N., De Geest, B.G., Goethals, J., Bajek, A., Dierckens, K., Bossier, P. & Vanrompay, D. (2018) Recombinant ferritin-H induces immunosuppression in European sea bass larvae (*Dicentrarchus labrax*) rather than immunostimulation and protection against a *Vibrio anguillarum* infection. *Veterinary Immunology and Immunopathology,* **204,** 19-27.

Yaacob, E.N., Goethals, J., Bajek, A., Dierckens, K., Bossier, P., De Geest, B.G. & Vanrompay, D. (2017) Preparation and Characterization of Alginate Microparticles Containing a Model Protein for Oral

Administration in Gnotobiotic European Sea Bass (*Dicentrarchus labrax*) Larvae. *Marine Biotechnology,* **19,** 391-400.

Yang, J., Chin, Y.C., Jiang, B., Yu, X., Zhu, G.-Z., Chen, Y., Barnard, J. & Mei, W. (2009) hnRNP I inhibits notch signaling and regulates intestinal epithelial homeostasis in the zebrafish. *PLoS Genetics*, **5**.

Yilmaz, E., Genc, M.A. & Genc, E. (2007) Effects of dietary mannan oligosaccharides on growth, body composition, and intestine and liver histology of rainbow trout, *Oncorhynchus mykiss*. *Israeli Journal of Aquaculture-Bamidgeh,* **59,** 182-188.



Chapter 8 GENERAL DISCUSSION

Although a market study falls outside the scope of this PhD research, it feels important to indicate that the current (economic) policy seems to offer opportunities for Dover sole aquaculture. In 2012, the European Union formulated a Blue Growth Strategy to promote sustainable and innovative growth and development in the European marine and maritime sector. Indeed, the European seas and coasts are considered to be drivers for the European economy, potentially harboring 5.4 million jobs and producing a gross added value of around EUR 500 billion per year (European Commission, 2017). The proposed strategy consists of different components, including the development of the aquaculture sector and increasing marine knowledge to promote the blue economy. These European initiatives also led to national investments in blue growth. Recently the Belgian Blue Cluster was initiated, an intense cooperation between private companies active in different sectors dedicated to developing economic activities linked to the sea and coastal areas. Such a cluster organization provides a good environment for starting up new aquaculture enterprises and help to accelerate growth and improve international competition (De Blauwe Cluster, 2018).

For the moment, successful Dover sole aquaculture is hampered by amongst others the limited knowledge on the basic biology of the species and consequently the problems in the aquaculture production cycle. It has been argued that Dover sole and the closely related Senegalese sole are very similar in a biological point of view (Dinis et al., 1999; Björndal et al., 2016; Imsland et al., 2003). Since the amount of literature on Senegalese sole increased enormously during the last decade (Björndal et al., 2016; Morais et al., 2016), extrapolation of these results to Dover sole might be tempting. However, both species show considerable differences. Not at least a difference in temperature was observed, including optimal spawning temperature (Devauchelle et al., 1987 ;Dinis et al., 1999; Anguis & Canavate, 2005) and optimal growth temperature (Deveauchelle et al., 1987; Canavate, 2005; Schram et al., 2006). This difference in temperature is also inextricably linked to a different susceptibility to diseases (reviewed in Imsland et al., 2003). Furthermore, Dover sole appears much less tolerant to high stocking densities (Schram et al., 2006) compared with Senegalese sole (Engrola et al., 2002). Nonetheless, few studies can be found comparing between both species. Therefore, it is not known to what extent or for which characteristics extrapolation between species is possible. Although it is theoretically possible to produce Dover sole, it appears expensive and therefore difficult to be economically profitable. This was illustrated in the past by the bankruptcy in 2010 of the Dutch IMARES spinoff 'Solea BV' (www.faillissementen.com, accessed on 02/01/2018). However, research on the cultivation of the species did not stop (e.g. Fry Marine and Stichting Zeeschelp, the Netherlands) and future projects might have a greater chance of success due to increased knowledge on the species and its production cycle. To guarantee its success, it might also be of great importance to act on the purchasing behaviour of the consumer towards aquaculture products. Fish consumption appears still

to be price sensitive in Western- European countries (European Commission, 2016), which might be disadvantageous for Dover sole aquaculture. However, an increased focus and overall positive attitude of European consumers towards sustainable aquaculture was reported (European Commission, 2016; Risius et al., 2017). On the other hand, it was stated that communication and labelling should continue to have a decisive impact on the buying behaviour (Pieniak et al., 2013; Risius et al., 2017) and elaborating on this aspect might help Dover sole aquaculture one step forward. However, as stated before, market studies belong to a different research discipline and only very limited information on the marketability of Dover sole was found. The above mentioned thoughts should therefore merely be seen as discussion items, rather than objective statements.

Nonetheless, the potential of Dover sole aquaculture is clear. As for any new aquaculture species, becoming an established value takes time and requires a lot of research on many aspects concerning both the market and the species. Indeed, the latter is where the results of this PhD research contribute to. In a first study, the ultrastructural morphology of the Dover sole egg was described whereupon we will elaborate in this general discussion section including future possibilities. Next, a housing and challenge model were pinpointed for Dover sole for the first time and both were implemented to evaluate various immunostimulants. In what follows, we will discuss the advantages and limitations of the state-of-the-art experimental models, further expansions on the model and possible fields of application. Furthermore, the use of immunostimulants in the larviculture will be considered. Last but not least, we will pose some reflections about ethics.

8.1 MORPHOLOGICAL STUDY

In chapter 4, adequate protocols for scanning (SEM) and transmission electron microscopy (TEM) of Dover sole eggs were established and the **ultrastructural morphology of the Dover sole eggs** was described. In a next step, these techniques may be used to investigate the impact of various treatments. Considering the disinfection treatments tested in chapter 5, it would be interesting to investigate the impact of the disinfection protocols on the morphology of the Dover sole egg. Indeed, decreased embryonic survival following disinfection might be linked to observable damage of the egg envelope or changes in the egg structure. Albeit in very low densities, bacterial cells were observed based on electron microscopy in our study. Large bacterial populations were also reported in other studies using SEM and TEM (Hansen & Olafsen, 1989; 1999; Morrison et al., 1999). Also bacterial breakdown of the outer layer of the egg envelope was suggested to impair hatching results (Lønning & Davenport, 1980). Investigating the effect of bacteria and egg disinfection on the ultrastructural morphology of the Dover sole egg might also shed light on the disinfection potential of future protocols.

Generally, teleosts are considered important indicators of environmental pollution. In particular, extensive knowledge on the impact of pollution on fish reproduction was published (reviewed in Devlin & Nagahama, 2002), primarily focusing on sex differentiation, gonad morphology, gametogenesis and sex phenotypes. Especially the process of fish oogenesis was reported to be sensitive to environmental pollutants and endocrine disrupting chemicals (e.g. estrogens), resulting in altered fish oocyte proteins (including egg envelope proteins) and vitellogenin (Arukwe et al., 1997; Arukwe & Goksøyr, 2003). Changes in egg envelope proteins might result in differences in thickness and strength of the envelope, affecting fertilization success and polyspermy prevention or the degree of embryonic protection during development (Arukwe et al., 1997; Arukwe & Goksøyr, 2003). The described protocols for electron microscopy make it possible to further investigate these parameters in Dover sole eggs. To include Dover sole eggs as biomarker of environmental pollution, characterization of the protein and glucoprotein composition of the egg envelope would be the next step, followed by investigating the impact of pollution on the oogenesis of this species. In the view of aquaculture production, endocrine regulation of the spawning might also result in changing egg envelope composition when the protocols are not sufficiently balanced. Therefore, alterations in the protein content of the egg envelope should be taken into account during the development of these protocols.

8.2 STATE-OF-THE-ART HOUSING AND CHALLENGE MODEL

8.2.1 Advantages and limitations

A cutting-edge **multiwell housing system** was pinpointed for Dover sole larvae (chapter 5). Indeed, the present innovative model may be the starting point for a large variety of future trials as exemplified by the implementation of the model in subsequent Dover sole larval experiments (chapter 6 and 7). This model has multiple advantages. As already discussed, housing the larvae individually has the advantage that the death of one individual has no impact on the adjacent one and less animals are needed since each well or well plate may be considered an independent replicate. Furthermore, housing the larvae in small wells and feeding them ad libitum starting from first feeding, increases the feed efficiency at mouth opening. The energy depletion observed in the wild or large hatchery tanks in which more energy is invested in searching and capturing the prey compared with the energy gain of the digested feed (Richard et al., 1991), is therefore much less present in larvae housed in the multiwell system.

Apart from the aforementioned benefits, we should acknowledge that the use of multiwell plates as housing method also may elicit some **critical remarks** that we can refute to a large extent. Since the wells contain a rather small static water mass, caution is needed to avoid accumulation of detrimental larval excretions or bacterial overgrowth. It is common knowledge that high nutrient concentrations and water detention facilitate microbial growth (Douillet & Langdon, 1994) and that (opportunistic)

pathogenic bacteria play an important role in larval mortality (Hansen & Olafsen, 1999; Bricknell & Dalmo, 2005; Austin & Austin, 2012). To remediate this, autoclaved seawater was used in the Dover sole housing model and well water was replaced every other day. Furthermore, eggs were disinfected at the start of the experiments, to avoid bacterial overload in the egg and larval stage. However, the virucidal effect of the disinfection protocol was not investigated, although an optimal protocol should kill viruses as well.

In the view of larval welfare, well plate movement was restricted to a minimum. Since larval survival was high, it may be stated that the absence of any form of water flow did not result in oxygen depletion. Presumably, the gas exchange in between well water and air remained adequate due to the small water mass in comparison with the large surface area. Also feeding intake was not impaired as the larvae were reared on live feed, which has the capacity to swim throughout the entire well. However, when adding other components to the well water, it is important to take the sinking and aggregation potential of the product into consideration. Aggregation of the substances, bacterial or fungal cells leads to sedimentation (Trunk et al., 2018) and heavy particles may sink to the bottom of the well. This will not only compromise ingestion by the larvae but also promote bacterial overgrowth or decreased water quality and should therefore be avoided (Silva, 2007). In the developed challenge model and prebiotic supplementation trials, no turbidity which might suggest bacterial aggregation was observed. Adding the potentially prebiotic component alginic acid to the water did result in minimal aggregation. However, prebiotic supplementation was performed via bioencapsulation in *Artemia* nauplii in aerated beakers, followed by rinsing of the *Artemia* before feeding to the larvae. Therefore, no impact of the aggregation of alginic acid was expected on the Dover sole larvae in the wells. Finally, it should be stated that the use of multiwell plate systems is ideally to perform small scale laboratory experiments. However, the experimental housing model might be less appropriate for large experiments including several thousands of larvae since exchanging well water is rather labour intensive and a large surface area is needed to set up the experiment. Indeed, to avoid larval stress, well plates should not be stacked but placed side by side on a dark surface in a temperature controlled room.

In the present PhD research, we did not succeed in developing a **gnotobiotic model** for Dover sole larvae. Although a large variety of disinfection protocols was tested, none of these resulted in consistently sterile eggs without reducing embryonic survival and hatching. As mentioned before, gnotobiotic models are excellent tools to investigate bacteria-host interactions with the complete control of the bacteriological status of the larvae. In this way, the experimental reproducibility is increased hence leading to more unambiguous results. However, in a first step, an axenic larvae need to be obtained and several drawbacks were reported concerning the use of these axenic animals.

Pathophysiological abnormalities of the gastrointestinal tract were observed in germfree zebrafish larvae compared with conventional animals (Rawls et al., 2004; Bates et al., 2006). The importance of the gut microbiome was already acknowledged in several animal species as germfree individuals displayed altered immune responses, organ development, metabolism and reproduction (Williams, 2014), hereby indicating that the gut microbiome is essential for normal development and immunity. Since the germfree larvae differ greatly from the conventional larvae and alterations in basic structures and processes were observed, utmost caution is needed when extrapolating the findings of these models to the conventionally kept individuals. In gnotobiotic models a selection of known bacteria are included. However, this is not a complete representation of the conventional microbial community and therefore similar drawbacks to the use of gnotobiotic larvae need to be taken into account. Verification as to whether the obtained results also apply to conventional systems therefore is a necessity.

In chapter 5, an experimental **challenge model** for Dover sole with *V. anguillarum* WT was developed, resulting in a larval mortality of 50 % at 7 days post-inoculation (DPI) (=17 days after hatching (DAH)) when added at a concentration of 10^7 CFU/ml. This challenge model was used to investigate the protective potential of probiotics administered via the water and the feed. Two separate experiments exploring the immunomodulating effect of prebiotics also adopted this model. Throughout all experiments, the mortality ranged between 50 and 85 %, with the highest mortality observed in the batches with the lowest survival in the control group (chapter 7). These results point towards the reproducibility of the model. Upon its application, one needs be cautious as various *in vitro* passages potentially impact the virulence of bacteria (Li et al., 2014). Furthermore, the storage and *in vitro* cultivation methods also may have a clear impact on virulence (Ishiguro et al., 1981; Michel & Garcia, 2003). This provides the rationale for us working with bacterial cells that were only passaged to a minimal extent.

All experiments were performed with fish eggs and larvae derived from broodstock maintained in captivity. However, all broodstock consisted of adult fish originally wild caught in the North Sea (the Netherlands). As mentioned in the introduction, **different Dover sole populations** in Europe were delineated. We may hypothesize that differences in genetic structure may impact the sensitivity of the larvae to *V. anguillarum* WT. Indeed, also in other fish species pathogen resistance was related to genetic differences, generally linked to variability at genes of the major histocompatibility complex (MHC) (Arkush et al., 2002; Dionne et al., 2007). Verification of the pinpointed challenge model with more southern Dover sole populations might be important before starting experiments using animals caught in these regions.

The innovative research tools developed during this PhD research ensure a standardized and controlled experimental set-up in laboratory trials. However, caution is needed when extrapolating the obtained results to **field** situations. Depending on the aim of the study, this 'field situation' may be the aquaculture facilities or the ocean. The latter defines an environment which is highly variable both in time and space whereas aquaculture settings change less quickly but other aspects such as intensification may largely impact the results. The standardized approach which is sought in the laboratory studies, is of major importance to increase the reproducibility as well as substantiate comparison between studies but at the same time the main drawback to extrapolate the results to the wild. Therefore, it is of major importance to verify the obtained results in larger trials mimicking the field situation to a better extent. Reversing this reasoning, it may be interesting to review the literature on former trials involving Dover sole larvae performed in larger tanks. The pinpointed research models might shed light on the underlying mechanisms causing the observed results.

As mentioned above, the multiwell housing system makes it possible to house Dover sole larvae individually. The well system is also used in toxicity tests with larvae in which each well is considered an independent replicate (OECD, 2013). However, prudence is called for when determining the experimental set-up and related statistical analysis. As presented in a clear way in the study of Lazic et al. (2018), **genuine replication** is only achieved when the biological, observational and experimental units are correctly defined. The biological unit (BU) is the entity/subject of the hypothesis, which was in our case the Dover sole larva. The observational unit (OU) is the entity on which the measurements or samples are taken, which was also the individual larva in our experiments. The experimental unit (EU) is defined as the entity that is assigned to the experimental treatment and may correspond to one BU, parts of a BU or a group of BUs (Lazic et al., 2018). In case the treatment is performed on individual larvae/wells, then these wells are considered the BU, EU and OU. Therefore, the wells can be treated as independent replicates. In the proposed toxicity tests of the OECD (OECD, 2013), treatments should be randomized across the wells and each well should be assigned to a different treatment and the wells are therefore correctly identified as independent replicates. However, in the challenge experiments performed during this PhD research, treatments were randomized and assigned at the level of the well plate and therefore the well plate is to be considered the EU. Treatments were not assigned to individual wells because the danger of cross-contamination was too high. The individual well should therefore be interpreted as repeated measurements, not replicates. Consequently, it is important to incorporate several well plates (= replicates) per treatment. For this reason, four well plates per treatment were included in all our experiments. Post-treatment, the use of statistical analysis embracing the inter plate variation could further determine whether individual wells or well plates should be interpreted as independent replicates in the following statistical analysis. In our

experiments, individual wells were determined as independent replicates during development of the challenge model and assessing probiotic supplementation (chapter 6). Well plates were indicated as independent replicates when assessing prebiotic supplementation (chapter 7).

Across all experiments, **egg and larval quality** varied widely. More than once, egg batches were withheld before the start of the experiments due to low fertilization rates or batch quality. Furthermore, it was observed that larval survival in the negative control group varied depending on the experiment. At 17 DAH, survival ranged between 76 % (chapter 6 – Harmfullness of the probiotic candidate) and 92 % (chapter 6 – Experimental infection model). At 21 DAH, survival ranged between 49 % (chapter 7 - Survival) and 80 % (chapter 6 – Probiotic protection). In chapter 5, the multiwell plate housing model was established and larvae were reared until 26 DAH. For 4 batches, survival ranged between 58 and 85 %. This variability in mortality between batches was already reported for larvae of different fish species, including Dover sole larvae (Lund, 2007; Bolle et al., 2012) and varying egg quality is one of the limiting factors for successful mass production of fish fry (Kjorskvik et al., 1990). Larval performance was linked to several factors, one of these being the biochemical composition of the nutrients derived during embryonic development and may therefore be dependent on the broodstock nutritional status during oogenesis (Sargent et al., 1999, Morais et al., 2004). Indeed, the importance of different levels of fatty acids (FA) in the feed was shown for many fish species. In Dover sole, oocyte development is a continuous process (Imsland et al., 2003) and the egg FA composition reflects feed composition of the broodstock prior to the breeding season (before and during vitellogenesis) (Devauchelle et al., 1987; Parma et al., 2013) and not close to egg maturation as was suggested (Imsland et al., 2003). Egg size decreases during the course of the spawning season and a smaller egg size was linked to increased mortality (Houghton et al., 1985; Rijnsdorp and Vingerhoed, 1994). However, this increased mortality was not linked to the FA composition, as it is possible to obtain a similar egg quality in terms of FA profile throughout the whole spawning season (Parma et al., 2013). In Dover sole, egg size was directly associated with larval size. Indeed, larvae hatched from larger eggs contained a larger yolk sac and were both longer and had deeper myotomes than those hatched from the smaller eggs. Furthermore, since more yolk was available at the time the jaw became functional, larvae hatching from larger eggs had less chance of starvation due to the inadequate predation success at first-feeding (Baynes & Howell, 1996). In view of the above, it may be of crucial importance not to include egg batches at the end of the spawning season in experimental studies, as egg size decreases during spawning and therefore egg quality may be impaired. Furthermore, one could suggest that screening of the eggs on size would be important to obtain an 'equal' batch, to minimize variation in mortality caused by differences in egg size. On the other hand, a mixed batch creates a condition that mimics better the field situation, both in nature as well as in aquaculture facilities. Considering the

described experimental housing system in which the larvae are housed individually and the death of one larva has no impact on the adjacent one, it might also be possible to only work with the surviving larvae, even if the egg or larval quality is very poor. However, since the underlying mechanisms of low quality are not completely known, it is also not possible to predict the effect on the surviving larvae. Indeed, we may hypothesize that these animals already have an impaired health and therefore react differently to a pathogen challenge or pro- and prebiotic treatment.

8.2.2 Further elaboration of its potential

The promising housing and infection models for Dover sole larvae offer a myriad of opportunities to study the impact of various (a)biotic components and a set of both direct and indirect parameters were already incorporated in the models. However, like any critical researcher, we see a lot of opportunities to elaborate the potential of the developed models. Depending on the objectives of the study, additional innovative parameters may be pinpointed and subsequently added to the research toolbox established to investigate Dover sole larval health and well-being.

The pinpointed experimental housing and infection models were implemented to investigate the effect of several immunostimulants on larval health. To our knowledge, these were the first studies in Dover sole evaluating both the direct (length, harmfulness, apoptosis) and indirect effect (survival following challenge) of pro- and prebiotic supplementation. In both experiments, larvae were reared until 21 DAH, including a period of 11 days post pathogen challenge. In future studies, it may be an added value to include **long term monitoring**. Indeed, rearing the larvae for a longer period of time would make it possible to include (the completion of) metamorphosis as a parameter. Indeed, as mentioned in the introduction, metamorphosis in flatfish is a very complex process, involving many morphological and physiological changes and resulting in the migration of the eye and 90° rotation of the body (Palazzi et al., 2006; Piccinetti et al., 2012). Due to the complexity of the process, larvae appear to be very sensitive to any stressor during this transition. Next to the morphological parameter, also differences in gene expression may be included. For the latter, genes involved in the metamorphosis of Dover sole were already determined (Ferraresso et al., 2013). Additionally, malpigmentation may only be observed in juveniles, hereby again stressing the need to maintain the larvae for a longer period of time. Increasing the monitoring period would also facilitate a longer period of pro- or prebiotic administration. Indeed, larval supplementation did not exceed 6 days in the Dover sole (chapter 6 and 7) but a positive impact following a short period of administration was observed for other fish larvae (Strand & Dalmo, 1997; Schaeck et al., 2016b; 2017c). However, in most studies on fish, a dietary immunostimulants feeding regime (with beneficial effects) varies between 1 to 10 weeks (probiotics: reviewed in Nayak, 2010a; prebiotics: reviewed in Ringø et al., 2010; 2012). The developed housing model in our study was only evaluated until 26 DAH. Rearing the larvae for **a longer period of time**

engenders two extra challenges. First, the use of larger rearing units should be evaluated. In first instance, 12 or 6 well plates may be used as the larger well size and corresponding water body would enable to keep the larvae for a longer period of time. When one has an even longer housing period in mind, small aquaria need to be applied. In these cases, the researcher needs to consider if the individual housing is still necessary to obtain the predetermined results and different scenarios may be proposed. Depending on the study, next to individual housing until the end, larvae may be housed individually until completion of metamorphosis, followed by group housing for the remaining period. In the latter case, larvae may be tagged if necessary, to allow an individual based observation. Secondly, keeping the larvae of a longer period of time would make it necessary to develop an adequate feeding regime. The present housing model included larval feeding with sterile *Artemia* nauplii every other day, starting from 6 DAH with good survival rates and apparently good health status (incl. good feeding behaviour). However, to meet the nutritional requirements for the juvenile or adult stage, weaning to dry feed needs to be established. Although weaning is regularly performed in Dover sole (Day et al., 1997; Imsland et al., 2003; Bonaldo et al., 2011; Parma et al., 2013), to our knowledge, this was not yet accomplished in a well plate system and is a challenge that needs to be tackled.

Housing the larvae individually also makes it possible to include **behaviour** as a parameter in future experiments. To our knowledge, impact on behaviour was not studied in previous experiments involving pro- or prebiotics. Increased resilience to stress following probiotic supplementation was documented for larvae of several roundfish such as seabass (Aerts et al., 2018) and juveniles of the flatfish Japanese flounder (Taoka et al., 2006). Also a decreased gene expression of Heat Shock Protein 70 was observed in Dover sole juveniles following 50 days of probiotic treatment, indicating a stress reduction (Avella et al., 2011) but no behavioural analyses were performed. However, behavioural changes linked to changed stress resilience might occur following treatment. In contrast to adult fish, larval behaviour is limited due to a still immature nervous system compared to older animals. However, the basal activity (locomotion), photomotor response (=response to changes in light intensity) or reactions to anxiety may already be assessed (Colwill & Creton, 2011a; 2011b). The multiwell housing system for Dover sole, may be considered a step forward in inclusion of sole behaviour in future toxicity testing. However, monitoring Dover sole larvae in this manner is not that straightforward. Indeed, Dover sole larvae exhibit low mobility, especially in these wells, impeding the evaluation of this parameter between treatments. Furthermore, no objective behavioural baseline parameters were published for this species, in contrast with zebrafish (Colwill & Creton, 2011a; 2011b; Kalueff et al., 2013). In a first step, the basic behaviour and normal responses to stimuli for Dover sole need to be established. In a next step, the effect of different treatments may be investigated. With the multiwell housing system, it is possible to house and monitor the larvae in the same environment, hereby

reducing stress prior to the behavioural analyses and maintaining the larvae individually throughout both the treatment and monitoring, hereby ensuring complete independent follow-up of the larvae.

Furthermore, exposure to immunostimulants or other compounds might affect the **swimming capacity**, one of the crucial factors for fish larvae determining survival in the wild. To evaluate this, the critical swimming speed (= the speed at which maximum oxygen uptake occurs; De Boeck et al., 2006; Liew et al., 2012) as well as fast start performance (important for escape and predation, Tudorache et al., 2008) may be measured. Depending on the objectives of the study, also this parameter may be evaluated and included as a parameter.

In all experiments, the effect of pro- and prebiotic administration on larval length was included in our experiments as an indicator of larval growth. Indeed, including this parameter is of major importance for the aquaculture sector as rapid growth is directly linked to faster marketing of the fish. Length measurements were performed immediately following euthanasia of the larvae and prior to fixation, since significant shrinkage and shape effects following different fixatives were reported for fish larvae (Nikolakakis et al., 2014). When expanding of the housing model to other experiments, including the effect of compounds or treatments prone to cause malformations (e.g. nutritional studies), it may be important to include not only length measurements but also **morphometric analyses** (=shape analyses). The inclusion of elaborate morphometric analyses would provide additional information on the effect of specific treatments on the overall shape of fish larvae that also might be an indicator of increased deformity rates. In hatcheries, detection of malformations at a very early age is crucial to predict economic losses due to deformities in a later stage. Generally, identification of malformations is based on skeletal morphology and therefore mineralized elements such as deformed or fused vertebrae. Since ossified structures are nearly absent in fish larvae and larvae show very little characteristics with distinguishable variation, more sensitive methods such as shape analyses need to be implemented (Nikolakakis et al., 2014). Morphometric analysis was already applied for different fish species including Dover sole larvae, in the latter to investigate the effect of pulse trawling on development (Desender et al., 2018). Furthermore, even more advanced shape analyses based on the outline-based method have been performed in larval studies on seabass (Nikolakakis et al., 2014; 2018) and European eel (*Anguilla anguilla*, De Meyer et al., 2015). However, to our knowledge, morphometric analyses were not included in any studies on pro- and prebiotic treatment on fish larvae.

Although much research attention was already paid to studying the beneficial effects of immunostimulants on fish larvae, the mode of action still is largely unknown and although a variety of immune responses were proposed, only a limited number of studies focus on this topic (Verschuere et al., 2000; Nayak, 2010a; Zhou & Wang, 2012; De et al., 2014). The use of probiotics to lower the **stress**

response in marine fish, was proposed for juvenile fish (Carnevali et al., 2006; Rollo et al., 2006; Hernandez et al., 2010; Varela et al., 2010) and recently also for larvae of seabass (Aerts et al., 2018). Following a stressful stimulus, fish present an endocrine stress response through activation of the hypothalamic-pituitary-interrenal (HPI) axis, followed by the release of in the blood (Aerts et al., 2015). In teleost fish, cortisol is the dominant glucocorticoid which is also involved in the regulation of several physiological processes, including growth, reproduction and immunoregulation (Mommsen et al., 1999; Vazzana et al., 2002; Blas et al., 2007). To verify a possible change in stress level, various methods may be applied. In adult fish, cortisol levels in the blood plasma of the fish are widely used to evaluate the effect of a stressor (Easy & Ross, 2010). However, the levels show a diurnal pattern (Oliveira et al., 2013) and blood sampling is an invasive method inducing stress by itself. Furthermore, blood sampling is not possible in small fish larvae. For the latter, specific methods need to be applied. In fish larvae, cortisol levels may only be determined by performing whole body analyses and this has per definition the disadvantage that the larvae need to be euthanized and no serial sampling may be performed (for example before and after stressor or before and after immunostimulant supplementation). Quantification is generally performed based on enzyme immunoassays (EIA) (Piccinetti et al., 2012; Tsalafouta et al., 2014). However, this technique is biased as cross-reactivity was observed between cortisol and other glucocorticoids as well as unknown substances with similar properties (Cook, 2012). In addition, this technique also requires a minimum amount of larval material, forcing researchers to pool different specimens, hereby blending the coping effect of the individual larvae. Recently, the glucorticoid profile of seabass larvae was established based on ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) (Aerts et al. 2018). This technique enables to quantify not only cortisol but also its precursors and phase I metabolites in the whole body of one single larva. To assess the impact of the probiotic *V. lentus*, the glucocorticoid profile of supplemented and gnotobiotic larvae was investigated. The administration of the probiotic candidates resulted in significantly decreased glucocorticoid baseline levels (Aerts et al., 2018). However, to define a possible improvement in stress resilience, the fish larvae need to be subjected to an acute stress challenge, hereby comparing glucocorticoid levels before and after acute stress. To our knowledge, hitherto, this was not yet performed in larval research concerning pro- or prebiotics. Modifications to the general stress response in fish larvae may also be detected by comparing the gene expression levels of the genes involved in the HPI axis between exposed and unexposed larvae (Palermo et al., 2011; Ferraresso et al., 2013; Samaras & Pavlidis, 2018; Tsalafouta et al., 2018). Also this technique requires a minimum amount of larval tissue and therefor the larvae are generally pooled. However, in contrast with EIA based analyses, no cross-reaction is observed and more than one aspect of the HPI pathway may be investigated. Where acute stress is known to increase the activity of the immune system and protect against pathogens, chronic stress will result in immune

suppression (Tort, 2011). Furthermore, the coping behaviour resulting from exposure to a stressor is expected to impose an allostatic cost, competing with the needs of the immune system and further causing immune suppression (Tort, 2011). It is therefore argued that chronic stressors in aquaculture facilities may contribute to an increased susceptibility to diseases in the industry. Hitherto, research on basal cortisol levels in fish larvae is scarce and levels vary around 7 ng/g in seabass and cod larvae between 8 and 10 DAH (King & Berlinsky, 2006; Tsalafouta et al., 2014; Aerts et al., 2018). Furthermore, a temporal pattern during ontogeny was established for both species (King & Berlinsky, 2006; Tsalafouta et al., 2014). However, no information on the basal cortisol levels of Dover sole is available and future research on this species might therefore tackle this research question. Furthermore, it would be interesting to examine the impact of the metamorphosis on the HPI axis. In contrast to the genes related to the innate and adaptive immune system of Dover sole larvae (Ferraresso et al., 2016), no gene expression profiles for the genes involved in the activation of the HPI-axis were investigated during larval development.

In the past, the genomic information on flatfish species remained limited since they are not systematically used as model organisms for basic nor (bio)medical research. However, the development of next-generation sequencing techniques largely increased the possibilities in genome research, as it allows to generate large amounts of DNA or RNA sequences in a quick and relatively inexpensive way. In flatfish (aquaculture) research, next-generation sequencing of the transcriptome under specific experimental or aquaculture production conditions would increase the knowledge of underlying mechanisms driving different responses to amongst others dietary modifications (including prebiotics) but also probiotic supplementation or pathogen challenges (Cerda & Manchado, 2012; Manchado et al., 2017). For both Dover and Senegalese sole, a *de novo* assemblage and characterization of the **transcriptome** was performed (Ferraresso et al., 2013; Benzekri et al., 2014) and data were made available in the public database Solea DB. A microarray tool was established and tested for Senegalese sole (Benzekri et al., 2014) including amongst others genes related to stress response, growth, larval development and the immune system. Recently, Ferraresso et al. (2016) developed a microarray for Dover sole larvae, focusing on the transcription of both innate and adaptive immune related genes. This study revealed high expression levels of genes involved in the innate immune system at hatching and first feeding, indicating early protection against pathogens. However, genes related to the adaptive immune system were only upregulated at metamorphosis. Including this microarray in future experiments on Dover sole larvae would shed light on the pathogenesis of *V. anguillarum* by implementing the developed challenge model. Furthermore, the impact of immunostimulant supplementation on the expression of genes involved in both the innate and adaptive immune system may be evaluated (Ferraresso et al., 2016). This microarray may be further

expanded with genes involved in larval development and metamorphosis (Ferraresso et al., 2013). Transcriptome analyses were already included in research concerning dietary changes in both Senegalese sole larvae (Jimenez-Fernandez et al., 2015; 2018; Bonacic et al., 2016) and in pathogen studies including juveniles (Alvarez-Torres et al., 2016), illustrating the potential of the technique.

8.2.3 Possible fields of application

The development of an embryo into a juvenile is considered to be a very sensitive phase as the fish needs to complete critical developmental processes (Hutchinson et al., 1998; Mohammed, 2013). These processes may easily be disrupted due to environmental changes, including exposure to toxic components (Crane et al., 2005). Indeed, embryonic and larval stages may be particularly vulnerable to toxic components as their metabolic rates are higher compared with adult fish. In addition, due to the undeveloped enzymatic system of the premature fish, detoxification is limited (Mohammed, 2013; Vasconcelos et al., 2010). Furthermore, when the exposure occurs via the water, the relatively high surface/volume ratio of the larvae ensures a rapid increase of the internal toxin concentration of the fish (Mohammed, 2013; Foekema et al., 2008). Resulting from the previous, **early life stage (ELS) tests** with fish are regularly applied in acute toxicity assays, standardized by the OECD guidelines (OECD, 2003). Besides the high sensitivity of the test, using ELS enables to house the larvae in multiwell plates, hereby reducing the available space needed to perform toxicity tests in comparison with the classical tank experiments on juvenile or adult fish. Consequently, a high amount of statistically independent replicates may be included, increasing the power of the experiments (Wedekind et al., 2007). However, as stated before, caution is needed to avoid pseudoreplication by determining the genuine biological and experimental unit. Furthermore, the model allows to include factors that might be of importance in the wild (e.g. pathogen challenge, temperature increases, stress resilience, etc.) (Wedekind et al., 2007). The test species proposed by the OECD comprise mainly freshwater and tropical roundfish species (e.g. zebrafish, medaka (*Oryzias latipes*)), which may not be representative for the fish population in the European waters (Wedekind et al., 2007). Furthermore, none of these species undergoes a very extensive metamorphosis which may be particularly sensitive and can be observed in flatfish species (Yamano, 2005). Hitherto, only one study investigated the possibility of including Dover sole as a test species in ELS toxicity tests, hereby examining the impact of PCB -126 (=dioxin like component) (Foekema et al., 2008). The resulting bio-assay covered the complete transition from embryo to juvenile, including metamorphosis and stresses the importance and possibilities of ELS toxicity tests with Dover sole. However, larvae were group housed in small beakers (100 ml) and the examined parameters were limited to survival (including time of death) and completion of metamorphosis, followed by the calculation of the LD_{50} value (=lethal dose resulting in 50 % mortality). The innovative housing and challenge model developed in this PhD dissertation may be crucial to

ensure the (increased) use of Dover sole in future ELS toxicity tests as a representative species for marine coldwater flatfish. Next to the previously described advantages of using a multiwell housing system in toxicity tests, a large variety of extra parameters, such as behaviour, may be included. In the last two decades, behavior assessment became a key component in toxicity tests, investigating the effect of pollutants, drugs and neurotoxins (Ahmad et al., 2012; Brooks, 2009; Legradi et al., 2018). However, these tests mainly focused on zebrafish as a model-organism for humans. Although behaviour has been included as a parameter in toxicity tests of adult and juvenile flatfish, including Dover sole (Alderson, 1979; Schram et al., 2013), no behaviour analyses were included in any ELS test with flatfish (Foekema et al., 2008; Mhadhbi & Beiras, 2012). Video recording of the fish is quite an efficient tool to better understand behavioral traits and allows an automated observation system. Multiwell plates were used extensively as it enables high-throughput behavioural monitoring of fish larvae (Ahmad et al., 2012; Zhou et al., 2014; Pantoja et al., 2017).

8.3 THE USE OF IMMUNOSTIMULANTS IN LARVICULTURE

In aquaculture settings, immunostimulants may be incorporated in the commercial feed by the manufacturers. However, this implies that the fish needs to be cultured on dry feed which may be difficult to achieve in larvae. Bath delivery of immunostimulants would facilitate the immunostimulation of yolk sac larvae that are not able to feed on or digest the dry feed. However, bath delivery might be expensive when used in flow-through water systems or may adversely affect the water quality by increasing the organic load in recirculation systems (Bricknell & Dalmo, 2005). In our studies, probiotic supplementation in Dover sole larvae was performed via the water and via the feed. Adding the probiotic via the water maximizes exposure before first-feeding (Reitan et al., 1998; Vine et al., 2006). In contrast, bio-encapsulation of the probiotic requires lower amounts of probiotic compounds and is therefore economically more profitable. To our knowledge, comparing both routes of delivery for probiotic administration was not yet done for fish larvae prior to this PhD research. Prebiotic supplementation was only performed via bio-encapsulation, to avoid aggregation and sedimentation of the prebiotic compounds in the static water of the wells.

The effect of immunostimulants in fish larvae was linked to considerable beneficial effects without detrimental consequences for the developing animal (Salvesen et al., 1999; Conceicaeo et al., 2001; Pedersen et al., 2004). However, some researchers indicated that the use of immunostimulants in young animals may result in abnormal development of the immune response as well as increased immune tolerance (Bricknell & Dalmo, 2005). This immune tolerance towards immunostimulation was observed in one study on carp (Nakamura et al., 1998). To our knowledge, this effect was not demonstrated in larval fish. However, the underlying mechanisms of immune tolerance are poorly

understood. To avoid tolerance, immunostimulants should preferably not be given continuously but in intervals (pulse feeding) or during a limited period of time (Bricknell & Dalmo, 2005). It may be stated that science-based studies concerning the impact of immunostimulants on the developing immune system are clearly lacking. The increasing amount of research concerning genomics and proteomics may shed more light on this issue as well as on the mode of action of immunostimulants in fish larvae. For Dover sole larvae, this research could benefit from the developed state-of-the-art models during this PhD research.

In larviculture, the first feeding tank is a complex ecosystem, enclosing a large variety of interactions. Indeed, the number of interactions (in) between larvae, (zoo)plankton, bacteria (in the organic matter or waterborne) and the cultivation system are tremendous (Vadstein et al., 2018). Although the negative impact of detrimental bacteria to larvae is widely known and recognized, the overall web of interactions is largely undefined. One of the first steps to unravel this complex tangle is to identify the present micro-organisms not in the least of the larvae (Vadstein et al., 2018). Since the gastro-intestinal tract was suggested as the main portal of entry for pathogenic bacteria in fish (Paolini et al., 2010), the microbial community of the gut is important to consider. In a next step, the effect of dietary components such as immunostimulants on the gut microbiota needs to be examined. During the last two decades, several studies focused on the alterations of the gut microbial community in fish larvae following probiotic (Ringø et al., 1996; Carnevali et al., 2006; Suzer et al., 2008; Lauzon et al., 2010a; 2010b; 2010c) or prebiotic supplementation (Skjermo et al., 2006). However, these studies were mainly based on culture dependent techniques. Due to the development of culture independent techniques such as Polymerase Chain Reaction Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analyses and next-generation sequencing, a more elaborate microbiome analysis may be performed. Although extensively studied in humans and terrestrial animals, microbiome analyses on fish are limited (reviewed in a.o. Tarnecki et al., 2017). Especially larval research would benefit from an increased research effort. The microbiome is influenced by many environmental factors such as temperature, salinity, season, geographic location, starvation and dietary shifts (Xia et al., 2014; Merrifield & Rodiles, 2015; Schmidt et al., 2015; Tarnecki et al., 2017; Egerton et al., 2018) and differences between adult and larvae of the same fish species were observed (Star et al., 2013; Bakke et al., 2015). Furthermore, both the composition as well as the diversity of the gut microbial community change during larval development in several fish species (Verner-Jeffreys et al., 2003; Palmer et al., 2007; McIntosh et al., 2008; Sun et al., 2013; Bakke et al., 2015). The limited knowledge on the gut microbiome in fish larvae is also illustrated by the contradicting statements concerning the influencing factors. Indeed, it was suggested that newly hatched larvae have a microbiome that reflects the microbial community of the environment (Nayak, 2010b; Austin, 2012). In contrast, several authors

suggested a very specific larval microbiome (Li et al., 2012; Sullam et al., 2012; Bakke et al., 2015) containing 'core components' associated with the host and independent of the (rearing) conditions (reviewed in Ghanbari et al., 2015). Depending on the study, the composition of the gut microbial community was dependent (Givens, 2012; Bakke et al. 2013; 2015) or independent of food-associated microbiota (Reid et al., 2009; Givens, 2012; Ingerslev et al., 2014a; 2014b). To our knowledge, no (gut) microbiome analyses were performed on Dover sole larvae although these analyses, preferably based on next-generation sequencing, would contribute to a better insight in the microbial community where the supplemented pro and prebiotics should manifest themselves. Including this information might help in selecting appropriate new probiotic and prebiotic candidates.

8.4 LAST BUT NOT LEAST: ETHICS

In addition to toxicity tests, behavioural analyses might also contribute to the ethical aspects of animal testing on fish larvae. Although sceptics still deny anything beyond reflex responses in fishes (Rose et al., 2014; Diggles et al., 2017), it is generally agreed that fish are capable of nociception and pain (Braithwaite & Huntingford, 2004; Brown, 2015; Sneddon et al., 2015; 2018a; 2018b). Recently, research on nociception in fish larvae had a breakthrough. Zebrafish larvae (5 DAH) respond in a similar way as adult fish to noxious stimuli, both in gene expression as well as behavioural changes (Steenbergen & Bardine, 2014; Curtright et al. 2015; Lopez-Luna et al. 2017a; 2017b; 2017c; 2017d). These findings highlight the importance of taking larval welfare into consideration when performing *in vivo* experiments. Increasing knowledge on larval behaviour would help researchers to recognize a range of signals predicting anxiety, stress or death. In a next step, humane endpoints might be identified as defining these requires extensive data on pain and suffering in fish. Possible actions that may be considered following the inclusion of humane endpoints in larval experiments are early termination of the experiment when enough relevant information is collected or the administration of analgesia if pain is not a parameter in the study (Sneddon, 2009). To our knowledge, hitherto, no such considerations were made in larval research. Currently, the few studies involving larval behaviour following noxious stimulation, primarily monitored swimming velocity and activity time (Steenbergen & Bardine, 2014; Curtright et al. 2015; Lopez-Luna et al. 2017a; 2017b; 2017c; 2017d). In contrast, for adult zebrafish, many different behavioural traits were described (Kalueff et al., 2013). Altered body coloration, feeding behaviour, lethargy or body curling were possibly linked to humane endpoints. The developed multiwell plate housing model for Dover sole might be a promising tool to further pinpoint these endpoints in (Dover sole) larval experiments, enabling individual behavioural analyses and improving animal welfare. For zebrafish larvae, the observed negative response to noxious stimulation was mitigated by the use of analgesic or pain-relieving drugs (Lopez-Luna et al., 2017a). However, analgesics in fish were only recently adopted and hitherto no valid protocols are available for fish

larvae. Consequently, analgesics were not included in our studies. Moreover, the fact that zebrafish larvae respond in a similar way to noxious stimuli as adults, does not automatically imply that they experience the same (level of) pain. More research is needed to ensure that these larvae meet all criteria for animal pain perception (Sneddon et al., 2014). Recognition of pain perception in fish larvae might also have implications for the European legislation on experimental animals. Currently, the legislation criterion is "independently feeding larval forms" (Directive 2010/63/EU) and therefore, larvae are considered experimental animals starting from the moment they become exogenous feeders. This definition does not take into account pain perception and including this parameter might change the age at which the larvae are considered to be experimental animals and therefore protected by legislation. During our experiments, prey hunting started around 4-5 DAH. Since pain perception was not yet investigated in Dover sole larvae, it is not possible to answer the question objectively whether larvae should be considered at a younger (or later) age as an experimental animal. Throughout the experiments carried out in the framework of the current PhD research, a change in behaviour during larval development was observed. Following hatching, larval behaviour seemed arbitrary and even the smallest disturbance (light, movement, physical contact) might result in mortality. Throughout the following days, this type of behaviour decreased and movement became more controlled and specific. Also disturbances became of less importance. For this reason, Dover sole larvae were translocated at 2 DAH to the 24-well plates in our studies, in contrast to seabass larvae which tolerated translocation best close to hatching (Schaeck et al., 2016a). Considering the random behaviour the first two days, one could argue that in this period, perception of the environment is indistinct and reaction is largely random or reflexive. A possible advancing of the age at which Dover sole larvae are considered experimental animals should therefore not exceed more than one or two days. However and importantly, these are merely some general thoughts and we might even say speculations based upon working experience with Dover sole larvae. In the view of legislation, such decisions need to be supported by fundamental research on basic larval behaviour at this small age as well as the confirmation of pain perception in Dover sole larvae. As stated above, the use of the multiwell housing system for Dover sole would facilitate this type of research.

8.5 CONCLUSION

The pinpointed housing and experimental infection model for vibriosis is to be considered a powerful tool to investigate the pathogenesis of *V. anguillarum* infections in Dover sole larvae. Furthermore, as was demonstrated in chapter 6 and 7, the model may be used to investigate several curative and preventive treatments and their mode of action. The inclusion of an even larger set of parameters would further enhance deepening of the larval studies on Dover sole. Next to aquaculture related

research, also biological research concerning the impact of environmental factors on the health of Dover sole larvae (as a model or target species) may be evaluated.

8.6 REFERENCES

Aerts, J., Metz, J.R., Ampe, B., Decostere, A., Flik, G. & De Saeger, S. (2015) Scales Tell a Story on the Stress History of Fish. *Plos One*, **10**.

Aerts, J., Schaeck, M., De Swaef, E., Ampe, B. & Decostere, A. (2018) *Vibrio lentus* as a probiotic candidate lowers glucocorticoid levels in gnotobiotic sea bass larvae. *Aquaculture,* **492**, 40-45.

Ahmad, F., Noldus, L.P.J.J., Tegelenbosch, R.a.J. & Richardson, M.K. (2012) Zebrafish embryos and larvae in behavioural assays. *Behaviour,* **149,** 1241-1281.

Alderson, R. (1979) Effect of Ammonia on the Growth of Juvenile Dover Sole, *Solea Solea* (L) and Turbot, *Scophthalmus Maximus* (L). *Aquaculture,* **17,** 291-309.

Alvarez-Torres, D., Podadera, A.M., Bejar, J., Bandin, I., Alonso, M.C. & Garcia-Rosado, E. (2016) Role of the IFN I system against the VHSV infection in juvenile Senegalese sole (*Solea senegalensis*). *Veterinary Research,* **47**.

Anguis, V. & Canavate, J.P. (2005) Spawning of captive Senegal sole (*Solea senegalensis*) under a naturally fluctuating temperature regime. *Aquaculture*, **243**, 133–145.Arkush, K.D., Giese, A.R., Mendonca, H.L., Mcbride, A.M., Marty, G.D. & Hedrick, P.W. (2002) Resistance to three pathogens in the endangered winter-run chinook salmon (*Oncorhynchus tshawytscha*): effects of inbreeding and major histocompatibility complex genotypes. *Canadian Journal of Fisheries and Aquatic Sciences,* **59,** 966-975.

Arukwe, A. & Goksoyr, A. (2003) Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. *Comp Hepatol,* **2,** 4.

Arukwe, A., Knudsen, F.R. & Goksoyr, A. (1997) Fish zona radiata (eggshell) protein: a sensitive biomarker for environmental estrogens. *Environ Health Perspect,* **105,** 418-422.

Austin, B. (2012) *Infectious disease in aquaculture: Prevention and control*. Elsevier, Cambridge, Massachusetts, United States.

Austin, B. & Austin, D.A. (2012) *Bacterial Fish Pathogens: disease of farmed and wild fish*. Fifth edition. Springer Dordrecht, the Netherlands.

Avella, M.A., Olivotto, I., Silvi, S., Ribecco, C., Cresci, A., Palermo, F., Polzonetti, A. & Carnevali, O. (2011) Use of *Enterococcus faecium* to improve common sole (*Solea solea*) larviculture. *Aquaculture,* **315,** 384-393.

Bakke, I., Coward, E., Andersen, T. & Vadstein, O. (2015) Selection in the host structures the microbiota associated with developing cod larvae (*Gadus morhua*). *Environmental Microbiology,* **17,** 3914-3924.

Bakke, I., Skjermo, J., Tu Anh, V. & Vadstein, O. (2013) Live feed is not a major determinant of the microbiota associated with cod larvae (*Gadus morhua*). *Environmental Microbiology Reports*, **5**, 537–548.

Bates, J.M., Mittge, E., Kuhlman, J., Baden, K.N., Cheesman, S.E. & Guillemin, K. (2006) Distinct signals from the microbiota promote different aspects of zebrafish gut differentiation. *Developmental Biology,* **297,** 374-386.

Baynes, S.M. & Howell, B.R. (1996) The influence of egg size and incubation temperature on the condition of *Solea solea* (L) larvae at hatching and first feeding. *Journal of Experimental Marine Biology and Ecology,* **199,** 59-77.

Benzekri, H., Armesto, P., Cousin, X., Rovira, M., Crespo, D., Merlo, M.A., Mazurais, D., Bautista, R., Guerrero-Fernandez, D., Fernandez-Pozo, N., Ponce, M., Infante, C., Zambonino, J.L., Nidelet, S., Gut, M., Rebordinos, L., Planas, J.V., Begout, M.L., Claros, M.G. & Manchado, M. (2014) De novo assembly, characterization and functional annotation of Senegalese sole (*Solea senegalensis*) and common sole (*Solea solea*) transcriptomes: integration in a database and design of a microarray. *Bmc Genomics,* **15**.

Blas, J., Bortolotti, G.R., Tella, J.L., Baos, R. & Marchant, T.A. (2007) Stress response during development predicts fitness in a wild, long lived vertebrate. *Proceedings of the National Academy of Sciences of the United States of America,* **104,** 8880-8884.

Bjørndal, T., Guillen, J. & Imsland, A. (2016) The potential of aquaculture sole production in Europe: Production costs and markets. *Aquaculture Economics & Management,* **20,** 109-129.

Bolle, L.J., De Jong, C.a.F., Bierman, S.M., Van Beek, P.J.G., Van Keeken, O.A., Wessels, P.W., Van Damme, C.J.G., Winter, H.V., De Haan, D. & Dekeling, R.P.A. (2012) Common Sole Larvae Survive High Levels of Pile-Driving Sound in Controlled Exposure Experiments. *Plos One,* **7**.

Bonacic, K., Campoverde, C., Sastre, M., Hachero-Cruzado, I., Ponce, M., Manchado, M., Estevez, A., Gisbert, E. & Morais, S. (2016) Mechanisms of lipid metabolism and transport underlying superior performance of Senegalese sole (*Solea senegalensis*, Kaup 1858) larvae fed diets containing n-3 polyunsaturated fatty acids. *Aquaculture,* **450,** 383-396.

Bonaldo, A., Parma, L., Bacliani, A., Serratore, P. & Gatta, P.P. (2011) Very early weaning of common sole (*Solea solea* L.) larvae by means of different feeding regimes and three commercial microdiets:

Influence on performances, metamorphosis development and tank hygiene. *Aquaculture,* **321,** 237-244.

Braithwaite, V.A. & Huntingford, F.A. (2004) Fish and welfare: do fish have the capacity for pain perception and suffering? *Animal Welfare,* **13,** S87-S92.

Bricknell, I. & Dalmo, R.A. (2005) The use of immunostimulants in fish larval aquaculture. *Fish & Shellfish Immunology,* **19,** 457-472.

Brooks, J.C. (2009) The Emergence of Behavioral Testing of Fishes to Measure Toxicological Effects. *Toxicological Research*, **25**, 9-15.

Brown, C. (2015) Fish intelligence, sentience and ethics. *Animal Cognition,* **18,** 1-17.

Canavate, J.P. (2005) Opciones del lenguado senegales *Solea senegalensis* Kaup, 1858 para diversificar la acuicultura marina. *Boletın del Instituto Espanol de Oceanografıa,* **21**, 147–154.

Carnevali, O., De Vivo, L., Sulpizio, R., Gioacchini, G., Olivotto, I., Silvi, S. & Cresci, A. (2006) Growth improvement by probiotic in European sea bass juveniles (*Dicentrarchus labrax*, L.), with particular attention to IGF-1, myostatin and cortisol gene expression. *Aquaculture,* **258,** 430-438.

Cerda, J. & Manchado, M. (2013) Advances in genomics for flatfish aquaculture. *Genes and Nutrition,* **8,** 5-17.

Colwill, R.M. & Creton, R. (2011) Imaging escape and avoidance behavior in zebrafish larvae. *Reviews in the Neurosciences,* **22,** 63-73.

Colwill, R.M. & Creton, R. (2011b) Locomotor behaviors in zebrafish (*Danio rerio*) larvae. *Behavioural Processes,* **86,** 222-229.

Conceicao, L.E.C., Skjermo, J., Skjak-Braek, G. & Verreth, J.a.J. (2001) Effect of an immunostimulating alginate on protein turnover of turbot (*Scophthalmus maximus* L.) larvae. *Fish Physiology and Biochemistry,* **24,** 207-212.

Cook, N.J. (2012) Review: Minimally invasive sampling media and the measurement of corticosteroids as biomarkers of stress in animals. *Canadian Journal of Animal Science,* **92,** 227-259.

Crane, H.M., Pickford, D.B., Hutchinson, T.H. & Brown, J.A. (2005) Effects of ammonium perchlorate on thyroid function in developing fathead minnows, Pimephales promelas. *Environmental Health Perspectives,* **113,** 396-401.

Curtright, A., Rosser, M., Goh, S., Keown, B., Wagner, E., Sharifi, J., Raible, D.W. & Dhaka, A. (2015) Modeling Nociception in Zebrafish: A Way Forward for Unbiased Analgesic Discovery. *Plos One,* **10**.

Day, O.J., Howell, B.R. & Jones, D.A. (1997) The effect of dietary hydrolysed fish protein concentrate on the survival and growth of juvenile Dover sole, *Solea solea* (L.), during and after weaning. *Aquaculture Research,* **28,** 911-921.

De, B.C., Meena, D.K., Behera, B.K., Das, P., Das Mohapatra, P.K. & Sharma, A.P. (2014) Probiotics in fish and shellfish culture: immunomodulatory and ecophysiological responses. *Fish Physiology and Biochemistry,* **40,** 921-971.

De Blauwe Cluster (2018) www.deblauwecluster.be (Accessed 05/01/2019)

De Boeck, G., Van Der Ven, K., Hattink, J. & Blust, R. (2006) Swimming performance and energy metabolism of rainbow trout, common carp and gibel carp respond differently to sublethal copper exposure. *Aquatic Toxicology,* **80,** 92-100.

De Meyer, J., Ide, C., Belpaire, C., Goemans, G. & Adriaens, D. (2015) Head shape dimorphism in European glass eels (*Anguilla anguilla*). *Zoology,* **118,** 413-423.

Desender, M., Dumolein, L., Duchateau, L., Adriaens, D., Delbare, D., Polet, H., Chiers, K. & Decostere, A. (2018) Pulse Trawling: The Impact of Pulsed Direct Current on Early Life Stages of Sole *Solea solea*. *North American Journal of Fisheries Management,* **38,** 432-438.

Devauchelle, N., Alexandre, J.C., Lecorre, N. & Letty, Y. (1987) Spawning of Sole (*Solea Solea*) in Captivity. *Aquaculture,* **66,** 125-147.

Devlin, R.H. & Nagahama, Y. (2002) Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture,* **208,** 191-364.

Diggles, B.K., Arlinghaus, R., Browman, H.I., Cooke, S.J., Cowx, I.G., Kasumyan, A.O., Key, B., Rose, J.D., Sawynok, W., Schwab, A., Skiftesvik, A.B., Stevens, E.D., Watson, C.A. & Wynne, C.D.L. (2017) Responses of larval zebrafish to low pH immersion assay. Comment on Lopez-Luna et al. *Journal of Experimental Biology,* **220,** 3191-3192.

Dinis, M.T., Ribeiro, L., Soares, F. & Sarasquete, C. (1999) A review on the cultivation potential of *Solea senegalensis* in Spain and in Portugal. *Aquaculture,* **176,** 27-38.

Dionne, M., Miller, K.M., Dodson, J.J., Caron, F. & Bernatchez, L. (2007) Clinal variation in mhc diversity with temperature: Evidence for the role of host-pathogen interaction on local adaptation in Atlantic salmon. *Evolution,* **61,** 2154-2164.

Douillet, P.A. & Langdon, C.J. (1994) Use of a Probiotic for the Culture of Larvae of the Pacific Oyster (*Crassostrea Gigas* Thunberg). *Aquaculture,* **119,** 25-40.

Easy, R.H. & Ross, N.W. (2010) Changes in Atlantic salmon *Salmo salar* mucus components following short- and long-term handling stress. *Journal of Fish Biology,* **77,** 1616-1631.

Engrola, S., Conceicao, L.E.C., Pereira, R. and Dinis, M.T. (2002) Growth and diet utilisation of sole, *Solea senegalensis* reared under different feeding regimes. In: Book of Abstracts of the 10th International Symposium on Nutrition and Feeding in Fish, 2–7 June 2002, Rhodes, Greece.

Egerton, S., Culloty, S., Whooley, J., Stanton, C. & Ross, R.P. (2018) The Gut Microbiota of Marine Fish. *Frontiers in Microbiology,* **9**.

European Commission (2016) The EU Consumer Habits – Final Report. Ed: Directorate-General for Maritime Affairs and Fisheries, Director-General, 1-64.

European Commission (2017) Report on the Blue Growth Strategy: Towards more sustainable growth and jobs in the blue economy. Staff Working Document, 128, 1-62.

Ferraresso, S., Bonaldo, A., Parma, L., Buonocore, F., Scapigliati, G., Gatta, P.P. & Bargelloni, L. (2016) Ontogenetic onset of immune-relevant genes in the common sole (*Solea solea*). *Fish & Shellfish Immunology,* **57,** 278-292.

Ferraresso, S., Bonaldo, A., Parma, L., Cinotti, S., Massi, P., Bargelloni, L. & Gatta, P.P. (2013) Exploring the larval transcriptome of the common sole (*Solea solea* L.). *Bmc Genomics,* **14**.

Foekema, E.M., Deerenberg, C.M. & Murk, A.J. (2008) Prolonged ELS test with the marine flatfish sole (*Solea solea*) shows delayed toxic effects of previous exposure to PCB 126. *Aquatic Toxicology,* **90,** 197-203.

Ghanbari, M., Kneifel, W. & Domig, K.J. (2015) A new view of the fish gut microbiome: Advances from next-generation sequencing. *Aquaculture,* **448,** 464-475.

Givens, C.E. (2012) A fish tale: comparison of the gut microbiome of 15 fish species and the influence of diet and temperature on its composition. PhD dissertation, Graduate Faculty of the University of Georgia, Georgia, United States.

Hansen, G.H. & Olafsen, J.A. (1989) Bacterial-Colonization of Cod (*Gadus Morhua* L) and Halibut (*Hippoglossus Hippoglossus*) Eggs in Marine Aquaculture. *Applied and Environmental Microbiology,* **55,** 1435-1446.

Hansen, G.H. & Olafsen, J.A. (1999) Bacterial interactions in early life stages of marine cold water fish. *Microbial Ecology,* **38,** 1-26.

Hernandez, L.H.H., Barrera, T.C., Mejia, J.C., Mejia, G.C., Del Carmen, M., Dosta, M., Andrade, R.D. & Sotres, J.a.M. (2010) Effects of the commercial probiotic *Lactobacillus casei* on the growth, protein content of skin mucus and stress resistance of juveniles of the Porthole livebearer *Poecilopsis gracilis* (Poecilidae). *Aquaculture Nutrition,* **16,** 407-411.

Houghton, R.G., Last, J.M. & Bromley, P.J. (1985) Fecundity and Egg Size of Sole (*Solea Solea* (L)) Spawning in Captivity. *Journal Du Conseil,* **42,** 162-165.

Hutchinson, T.H., Scholz, N. & Guhl, W. (1998) Analysis of the ECETOC aquatic toxicity (EAT) database - IV - Comparative toxicity of chemical substances to freshwater versus saltwater organisms. *Chemosphere,* **36,** 143-153.

Imsland, A.K., Foss, A., Conceicao, L.E.C., Dinis, M.T., Delbare, D., Schram, E., Kamstra, A., Rema, P. & White, P. (2003) A review of the culture potential of *Solea solea* and *S. senegalensis*. *Reviews in Fish Biology and Fisheries,* **13,** 379-407.

Ingerslev, H.C., Jorgensen, L.V., Strube, M.L., Larsen, N., Dalsgaard, I., Boye, M. & Madsen, L. (2014a) The development of the gut microbiota in rainbow trout (*Oncorhynchus mykiss*) is affected by first feeding and diet type. *Aquaculture,* **424,** 24-34.

Ingerslev, H.C., Strube, M.L., Jorgensen, L.V., Dalsgaard, I., Boye, M. & Madsen, L. (2014b) Diet type dictates the gut microbiota and the immune response against *Yersinia ruckeri* in rainbow trout (*Oncorhynchus mykiss*). *Fish & Shellfish Immunology,* **40,** 624-633.

Ishiguro, E.E., Kay, W.W., Ainsworth, T., Chamberlain, J.B., Austen, R.A., Buckley, J.T. & Trust, T.J. (1981) Loss of Virulence during Culture of *Aeromonas Salmonicida* at High-Temperature. *Journal of bacteriology,* **148,** 333-340.

Jimenez-Fernandez, E., Ponce, M., Rodriguez-Rua, A., Manchado, M. & Fernandez-Diaz, C. (2018) Assessing the role of vitamin C and iron in early larvae stages of *Solea senegalensis* fed enriched Artemia. *Aquaculture,* **488,** 145-154.

Jimenez-Fernandez, E., Ponce, M., Rodriguez-Rua, A., Zuasti, E., Manchado, M. & Fernandez-Diaz, C. (2015) Effect of dietary vitamin C level during early larval stages in Senegalese sole (*Solea senegalensis*). *Aquaculture,* **443,** 65-76.

Kalueff, A.V., Gebhardt, M., Stewart, A.M., Cachat, J.M., Brimmer, M., Chawla, J.S., Craddock, C., Kyzar, E.J., Roth, A., Landsman, S., Gaikwad, S., Robinson, K., Baatrup, E., Tierney, K., Shamchuk, A., Norton,

W., Miller, N., Nicolson, T., Braubach, O., Gilman, C.P., Pittman, J., Rosemberg, D.B., Gerlai, R., Echevarria, D., Lamb, E., Neuhauss, S.C.F., Weng, W., Bally-Cuif, L., Schneider, H. & Znrc (2013) Towards a Comprehensive Catalog of Zebrafish Behavior 1.0 and Beyond. *Zebrafish,* **10,** 70-86.

King, W. & Berlinsky, D.L. (2006) Whole-body corticosteroid and plasma cortisol concentrations in larval and juvenile Atlantic cod *Gadus morhua* L. following acute stress. *Aquaculture Research,* **37,** 1282-1289.

Kjorsvik, E., Mangorjensen, A. & Holmefjord, I. (1990) Egg Quality in Fishes. *Advances in Marine Biology,* **26,** 71-113.

Lauzon, H.L., Gudmundsdottir, S., Petursdottir, S.K., Reynisson, E., Steinarsson, A., Oddgeirsson, M., Bjornsdottir, R. & Gudmundsdottir, B.K. (2010a) Microbiota of Atlantic cod (*Gadus morhua* L.) rearing systems at pre- and posthatch stages and the effect of different treatments. *Journal of Applied Microbiology,* **109,** 1775-1789.

Lauzon, H.L., Gudmundsdottir, S., Steinarsson, A., Oddgeirsson, M., Martinsdottir, E. & Gudmundsdottir, B.K. (2010b) Impact of probiotic intervention on microbial load and performance of Atlantic cod (*Gadus morhua* L.) juveniles. *Aquaculture,* **310,** 139-144.

Lauzon, H.L., Gudmundsdottir, S., Steinarsson, A., Oddgeirsson, M., Petursdottir, S.K., Reynisson, E., Bjornsdottir, R. & Gudmundsdottir, B.K. (2010c) Effects of bacterial treatment at early stages of Atlantic cod (*Gadus morhua* L.) on larval survival and development. *Journal of Applied Microbiology,* **108,** 624-632.

Lazic, S.E., Clarke-Williams, C.J. & Munafo, M.R. (2018) What exactly is 'N' in cell culture and animal experiments? *Plos Biology,* **16**.

Legradi, J.B., Di Paolo, C., Kraak, M.H.S., Van Der Geest, H.G., Schymanski, E.L., Williams, A.J., Dingemans, M.M.L., Massei, R., Brack, W., Cousin, X., Begout, M.L., Van Der Oost, R., Carion, A., Suarez-Ulloa, V., Silvestre, F., Escher, B.I., Engwall, M., Nilen, G., Keiter, S.H., Pollet, D., Waldmann, P., Kienle, C., Werner, I., Haigis, A.C., Knapen, D., Vergauwen, L., Spehr, M., Schulz, W., Busch, W., Leuthold, D., Scholz, S., Vom Berg, C.M., Basu, N., Murphy, C.A., Lampert, A., Kuckelkorn, J., Grummt, T. & Hollert, H. (2018) An ecotoxicological view on neurotoxicity assessment. *Environmental Sciences Europe,* **30**.

Li, X., Defoirdt, T., Yang, Q., Laureau, S., Bossier, P. & Dierckens, K. (2014) Host-induced increase in larval sea bass mortality in a gnotobiotic challenge test with *Vibrio anguillarum*. *Diseases of Aquatic Organisms,* **108,** 211-216.

Li, X.M., Yu, Y.H., Feng, W.S., Yan, Q.Y. & Gong, Y.C. (2012) Host Species as a Strong Determinant of the Intestinal Microbiota of Fish Larvae. *Journal of Microbiology,* **50,** 29-37.

Liew, H.J., Sinha, A.K., Mauro, N., Diricx, M., Blust, R. & De Boeck, G. (2012) Fasting goldfish, *Carassius auratus,* and common carp, *Cyprinus carpio*, use different metabolic strategies when swimming. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology,* **163,** 327-335.

Lønning, S. & Davenport, J. (1980) The Swelling Egg of the Long Rough Dab, *Hippoglossoides Platessoides Limandoides* (Bloch). *Journal of Fish Biology,* **17,** 359-378.

Lopez -Luna, J., Al-Jubouri, Q., Al-Nuaimy, W. & Sneddon, L.U. (2017a) Impact of analgesic drugs on the behavioural responses of larval zebrafish to potentially noxious temperatures. *Applied Animal Behaviour Science,* **188,** 97-105.

Lopez-Luna, J., Al-Jubouri, Q., Al-Nuaimy, W. & Sneddon, L.U. (2017b) Impact of stress, fear and anxiety on the nociceptive responses of larval zebrafish. *Plos One,* **12**.

Lopez-Luna, J., Al-Jubouri, Q., Al-Nuaimy, W. & Sneddon, L.U. (2017c) Reduction in activity by noxious chemical stimulation is ameliorated by immersion in analgesic drugs in zebrafish. *Journal of Experimental Biology,* **220,** 1451-1458.

Lopez-Luna, J., Canty, M.N., Al-Jubouri, Q., Al-Nuaimy, W. & Sneddon, L.U. (2017d) Behavioural responses of fish larvae modulated by analgesic drugs after a stress exposure. *Applied Animal Behaviour Science,* **195,** 115-120.

Lund, I. (2007) The impact of dietary fatty acids in common sole larval (*Solea solea* L.) nutrition. PhD dissertation, Department of Aquaculture, Danish Technical University, Danmark.

Manchado, M., Benzekri, H., Seoane, P., Bautista, R., Sanchez, J.J., Cousin, X., Planas, J.V., Rebordinos, L. & Claros, M.G. (2017) Development of Genomic Tools in Senegalese Sole: Transcriptome Assembly, Annotated Database, Microarray and Genome Draft. *Aquaculture,* **472,** 160-161.

McIntosh, D., Ji, B., Forward, B.S., Puvanendran, V., Boyce, D. & Ritchie, R. (2008) Culture-independent characterization of the bacterial populations associated with cod (*Gadus morhua* L.) and live feed at an experimental hatchery facility using denaturing gradient gel electrophoresis. *Aquaculture,* **275,** 42-50.

Merrifield, D.L. & Rodiles, A. (2015) The fish microbiome and its interactions with mucosal tissues. In: Mucosal Health in Aquaculture. Ed: Beck, B. & Peatman, E. Academic Press, Cambridge, Massachusetts, United States, 273-295.

Mhadhbi, L. & Beiras, R. (2012) Acute Toxicity of Seven Selected Pesticides (Alachlor, Atrazine, Dieldrin, Diuron, Pirimiphos-Methyl, Chlorpyrifos, Diazinon) to the Marine Fish (Turbot, *Psetta maxima*) (Retracted article. See vol. 227, pg. 275, 2016). *Water Air and Soil Pollution,* **223,** 5917-5930.

Michel, C. & Garcia, C. (2003) Virulence stability in *Flavobacterium psychrophilum* after storage and preservation according to different procedures. *Veterinary Research,* **34,** 127-132.

Mohammed, A. (2013) Why are Early Life Stages of Aquatic Organisms more Sensitive to Toxicants than Adults? In: *New Insights into Toxicity and Drug Testing*, Sivakumar Gowder, IntechOpen.

Mommsen, T.P., Vijayan, M.M. & Moon, T.W. (1999) Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries,* **9,** 211-268.

Morais, S. , Aragão, C. , Cabrita, E. , Conceição, L. E., Constenla, M. , Costas, B. , Dias, J. , Duncan, N. , Engrola, S. , Estevez, A. , Gisbert, E. , Mañanós, E. , Valente, L. M., Yúfera, M. & Dinis, M. T. (2016) New developments and biological insights into the farming of *Solea senegalensis* reinforcing its aquaculture potential. *Reviews in Aquaculture*, **8,** 227-263.

Morais, S., Narciso, L., Dores, E. & Pousao-Ferreira, P. (2004) Lipid enrichment for Senegalese sole (*Solea senegalensis*) larvae: effect on larval growth, survival and fatty acid profile. *Aquaculture International,* **12,** 281-298.

Morrison, C., Bird, C., O'neil, D., Leggiadro, C., Martin-Robichaud, D., Rommens, M. & Waiwood, K. (1999) Structure of the egg envelope of the haddock, *Melanogrammus aeglefinus*, and effects of microbial colonization during incubation. *Canadian Journal of Zoology-Revue Canadienne De Zoologie,* **77,** 890-901.

Nakamura, O., Suzuki, Y. & Aida, K. (1998) Humoral immune response against orally administered human gamma globulin in the carp. *Fisheries Science,* **64,** 558-562.

Nayak, S.K. (2010a) Probiotics and immunity: A fish perspective. *Fish & Shellfish Immunology,* **29,** 2-14.

Nayak, S.K. (2010b) Role of gastrointestinal microbiota in fish. *Aquaculture Research,* **41,** 1553-1573.

Nikolakakis, S., Bossier, P., Kanlis, G., Dierckens, K. & Adriaens, D. (2014) Protocol for quantitative shape analysis of deformities in early larval European seabass *Dicentrarchus labrax*. *Journal of Fish Biology,* **84,** 206-224.

Nikolakakis, S., Dierckens, K., Bossier, P. & Adriaens, D. (2018) Phenotypic effects of antibiotic-induced axenity and egg disinfection in early larval European seabass (*Dicentrarchus labrax* L.). *Aquaculture Research,* **49,** 2536-2552.

OECD (2013) Test No. 236: Fish embryo acute toxicity (FET) test, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.

Oliveira, C.C.V., Aparicio, R., Blanco-Vives, B., Chereguini, O., Martin, I. & Sanchez-Vazquez, F.J. (2013) Endocrine (plasma cortisol and glucose) and behavioral (locomotor and self-feeding activity) circadian rhythms in Senegalese sole (*Solea senegalensis* Kaup 1858) exposed to light/dark cycles or constant light. *Fish Physiology and Biochemistry,* **39,** 479-487.

Palazzi, R., Richard, J., Bozzato, G. & Zanella, L. (2006) Larval and juvenile rearing of common sole *(Solea solea* L.) in the Northern Adriatic (Italy). *Aquaculture,* **255,** 495-506.

Palermo, F.A., Mosconi, G., Avella, M.A., Carnevali, O., Verdenelli, M.C., Cecchini, C. & Polzonetti-Magni, A.M. (2011) Modulation of cortisol levels, endocannabinoid receptor 1A, proopiomelanocortin and thyroid hormone receptor alpha mRNA expressions by probiotics during sole (*Solea solea*) larval development. *General and Comparative Endocrinology,* **171,** 293-300.

Palmer, P.J., Burke, M.J., Palmer, C.J. & Burke, J.B. (2007) Developments in controlled green-water larval culture technologies for estuarine fishes in Queensland, Australia and elsewhere. *Aquaculture,* **272,** 1-21.

Pantoja, C., Hoagland, A., Carroll, E., Schoppik, D. & Isacoff, E.Y. (2017) Measuring Behavioral Individuality in the Acoustic Startle Behavior in Zebrafish. *Bio-protocol,* **7**.

Paolini, A., Magi, G.E., Gennari, L., Egidi, C., Torresi, M., Vallerani, M., Berti, M., Pavone, A. & Giorgetti, G. (2010). Severe mortality in common sole (*Solea solea*) at post-weaning stage following environmental stress. *Bulletin of the European Association of Fish Pathologists,* **30**, 160-169.

Parma, L., Bonaldo, A., Massi, P., Yufera, M., Martinez-Rodriguez, G. & Gatta, P.P. (2013) Different early weaning protocols in common sole (*Solea solea* L.) larvae: Implications on the performances and molecular ontogeny of digestive enzyme precursors. *Aquaculture,* **414,** 26-35.

Pedersen, G.M., Gildberg, A. & Olsen, R.L. (2004) Effects of including cationic proteins from cod milt in the feed to Atlantic cod (*Gadus morhua*) fry during a challenge trial with *Vibrio anguillarum*. *Aquaculture,* **233,** 31-43.

Piccinetti, C.C., Ricci, L.A., Tokle, N., Radaelli, G., Pascoli, F., Cossignani, L., Palermo, F., Mosconi, G., Nozzi, V., Raccanello, F. & Olivotto, I. (2012) Malnutrition may affect common sole (*Solea solea* L.) growth, pigmentation and stress response: Molecular, biochemical and histological implications. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology,* **161,** 361-371.

Pieniak, Z., Vanhonacker, F. & Verbeke, W. (2013) Consumer knowledge and use of information about fish and aquaculture. *Food Policy,* **40,** 25-30.

Rawls, J.F., Samuel, B.S. & Gordon, J.I. (2004) Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proceedings of the National Academy of Sciences of the United States of America,* **101,** 4596-4601.

Reid, H.I., Treasurer, J.W., Adam, B. & Birkbeck, T.H. (2009) Analysis of bacterial populations in the gut of developing cod larvae and identification of *Vibrio logei*, *Vibrio anguillarum* and *Vibrio splendidus* as pathogens of cod larvae. *Aquaculture,* **288,** 36-43.

Reitan, K.I., Natvik, C.M. & Vadstein, O. (1998) Drinking rate, uptake of bacteria and microalgae in turbot larvae. *Journal of Fish Biology,* **53,** 1145-1154.

Richard, P., Bergeron, J.P., Boulhic, M., Galois, R. & Personleruyet, J. (1991) Effect of Starvation on Rna, DNA and Protein-Content of Laboratory-Reared Larvae and Juveniles of *Solea Solea*. *Marine Ecology Progress Series,* **72,** 69-77.

Rijnsdorp, A.D. & Vingerhoed, B. (1994) The Ecological Significance of Geographical and Seasonal Differences in Egg Size in Sole *Solea Solea* (L). *Netherlands Journal of Sea Research,* **32,** 255-270.

Ringø, E., Birkbeck, T.H., Munro, P.D., Vadstein, O. & Hjelmeland, K. (1996) The effect of early exposure to *Vibrio pelagius* on the aerobic bacterial flora of turbot, *Scophthalmus maximus* (L) larvae. *Journal of Applied Bacteriology,* **81,** 207-211.

Ringø, E., Olsen, R.E., Gifstad, T.O., Dalmo, R.A., Amlund, H., Hemre, G.I. & Bakke, A.M. (2010) Prebiotics in aquaculture: a review. *Aquaculture Nutrition,* **16,** 117-136.

Ringø, E., Olsen, R.E., Vecino, J.L.G., Wadsworth, S. & Song, S.K. (2012) Use of immunostimulants and nucleotides in aquaculture: a review. *Journal of Marine Science: Research and Development,* **2**, 104.

Risius, A., Janssen, M. & Hamm, U. (2017) Consumer preferences for sustainable aquaculture products: Evidence from in-depth interviews, think aloud protocols and choice experiments. *Appetite,* **113,** 246-254.

Rollo, A., Sulpizio, R., Nardi, M., Silvi, S., Orpianesi, C., Caggiano, M., Cresci, A. & Carnevali, O. (2006) Live microbial feed supplement in aquaculture for improvement of stress tolerance. *Fish Physiology and Biochemistry,* **32,** 167-177.

Rose, J.D., Arlinghaus, R., Cooke, S.J., Diggles, B.K., Sawynok, W., Stevens, E.D. & Wynne, C.D.L. (2014) Can fish really feel pain? *Fish and Fisheries,* **15,** 97-133.

Salvesen, I., Skjermo, J. & Vadstein, O. (1999) Growth of turbot (*Scophthalmus maximus* L.) during first feeding in relation to the proportion of r/K-strategists in the bacterial community of the rearing water. *Aquaculture,* **175,** 337-350.

Samaras, A. & Pavlidis, M. (2018) Regulation of divergent Cortisol responsiveness in European sea bass, *Dicentrarchus labrax* L. *Plos One,* **13**.

Sargent, J., McEvoy, L., Estevez, A., Bell, G., Bell, M., Henderson, J. & Tocher, D. (1999) Lipid nutrition of marine fish during early development: current status and future directions. *Aquaculture*, **179**, 217-229.

Schaeck, M., De Swaef, E., Van Den Broeck, W., Van Nevel, S., Boon, N., De Geyter, N., Morent, R., Demeestere, K., Duchateau, L., Coulombet, C., Haesebrouck, F. & Decostere, A. (2016a) Germ-free sea bass *Dicentrarchus labrax* larval model: a valuable tool in the study of host-microbe interactions. *Diseases of Aquatic Organisms,* **117,** 177-185.

Schaeck, M., Duchateau, L., Van Den Broeck, W., Van Trappen, S., De Vos, P., Coulombet, C., Boon, N., Haesebrouck, F. & Decostere, A. (2016b) *Vibrio lentus* protects gnotobiotic sea bass (*Dicentrarchus labrax* L.) larvae against challenge with *Vibrio harveyi*. *Veterinary Microbiology,* **185,** 41-48.

Schaeck, M., Reyes-Lopez, F.E., Vallejos-Vidal, E., Van Cleemput, J., Duchateau, L., Van Den Broeck, W., Tort, L. & Decostere, A. (2017) Cellular and transcriptomic response to treatment with the probiotic candidate *Vibrio lentus* in gnotobiotic sea bass (*Dicentrarchus labrax*) larvae. *Fish & Shellfish Immunology,* **63,** 147-156.

Schmidt, V.T., Smith, K.F., Melvin, D.W. & Amaral-Zettler, L.A. (2015) Community assembly of a euryhaline fish microbiome during salinity acclimation. *Molecular Ecology,* **24,** 2537-2550.

Schram, E., Van der Heul, J.W., Kamstra, A. & Verdegem M.C.J. Stocking density-dependent growth of Dover sole (*Solea solea*). *Aquaculture*, **252**, 339–247.

Schram, E., Bierman, S., Teal, L.R., Haenen, O., Van De Vis, H. & Rijnsdorp, A.D. (2013) Thermal Preference of Juvenile Dover Sole (*Solea solea*) in Relation to Thermal Acclimation and Optimal Growth Temperature. *Plos One,* **8**.

Silva, P.F. (2007) Gnotobiotic growth model system for *Dicentrarchus labrax* larvae. Msc thesis, University of Algarve, Portugal.

Skjermo, J., Storseth, T.R., Hansen, K., Handa, A. & Oie, G. (2006) Evaluation of beta-(1 -> 3, 1 -> 6)-glucans and high-M alginate used as immunostimulatory dietary supplement during first feeding and weaning of Atlantic cod (*Gadus morhua* L.). *Aquaculture,* **261,** 1088-1101.

Sneddon, L.U. (2009) Pain Perception in Fish: Indicators and Endpoints. *Ilar Journal,* **50,** 338-342.

Sneddon, L.U. (2015) Pain in aquatic animals. *Journal of Experimental Biology,* **218,** 967-976.

Sneddon, L.U. (2018a) Comparative Physiology of Nociception and Pain. *Physiology,* **33,** 63-73.

Sneddon, L.U., Lopez-Luna, J., Wolfenden, D.C.C., Leach, M.C., Valentim, A.M., Steenbergen, P.J., Bardine, N., Currie, A.D., Broom, D. M. & Brown, C. (2018b) Fish sentience denial: Muddying the waters. *Animal Sentience,* **115.**

Sneddon, L.U., Elwood, R.W., Adamo, S.A. & Leach, M.C. (2014) Defining and assessing animal pain. *Animal Behaviour,* **97,** 201-212.

Star, B., Haverkamp, T.H.A., Jentoft, S. & Jakobsen, K.S. (2013) Next generation sequencing shows high variation of the intestinal microbial species composition in Atlantic cod caught at a single location. *Bmc Microbiology,* **13**.

Steenbergen, P.J. & Bardine, N. (2014) Antinociceptive effects of buprenorphine in zebrafish larvae: An alternative for rodent models to study pain and nociception? *Applied Animal Behaviour Science,* **152,** 92-99.

Strand, H.K. & Dalmo, R.A. (1997) Absorption of immunomodulating beta(1,3)-glucan in yolk sac larvae of Atlantic halibut, *Hippoglossus hippoglossus* (L). *Journal of Fish Diseases,* **20,** 41-49.

Sullam, K.E., Essinger, S.D., Lozupone, C.A., O'connor, M.P., Rosen, G.L., Knight, R., Kilham, S.S. & Russell, J.A. (2012) Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. *Molecular Ecology,* **21,** 3363-3378.

Sun, Y.Z., Yang, H.L., Huang, K.P., Ye, J.D. & Zhang, C.X. (2013) Application of autochthonous *Bacillus* bioencapsulated in copepod to grouper *Epinephelus coioides* larvae. *Aquaculture,* **392,** 44-50.

Suzer, C., Coban, D., Kamaci, H.O., Saka, S., Firat, K., Otgucuoglu, O. & Kucuksari, H. (2008) *Lactobacillus* spp. bacteria as probiotics in gilthead sea bream (*Sparus aurata*, L.) larvae: Effects on growth performance and digestive enzyme activities. *Aquaculture,* **280,** 140-145.

Taoka, Y., Maeda, H., Jo, J.Y., Jeon, M.J., Bai, S.C., Lee, W.J., Yuge, K. & Koshio, S. (2006) Growth, stress tolerance and non-specific immune response of Japanese flounder *Paralichthys olivaceus* to probiotics in a closed recirculating system. *Fisheries Science,* **72,** 310-321.

Tarnecki, A.M., Burgos, F.A., Ray, C.L. & Arias, C.R. (2017) Fish intestinal microbiome: diversity and symbiosis unravelled by metagenomics. *Journal of Applied Microbiology,* **123,** 2-17.

Tort, L. (2011) Stress and immune modulation in fish. *Developmental and Comparative Immunology,* **35,** 1366-1375.

Trunk, T., Khalil, H.S. & Leo, J.C. (2018) Bacterial autoaggregation. *Aims Microbiology,* **4,** 140-164.

Tsalafouta, A., Papandroulakis, N., Gorissen, M., Katharios, P., Flik, G. & Pavlidis, M. (2014) Ontogenesis of the HPI axis and molecular regulation of the cortisol stress response during early development in *Dicentrarchus labrax*. *Scientific Reports,* **4**.

Tsalafouta, A., Sarropoulou, E., Papandroulakis, N. & Pavlidis, M. (2018) Characterization and Expression Dynamics of Key Genes Involved in the Gilthead Sea Bream (*Sparus aurata*) Cortisol Stress Response during Early Ontogeny. *Marine Biotechnology,* **20,** 611-622.

Tudorache, C., Viaene, P., Blust, R., Vereecken, H. & De Boeck, G. (2008) A comparison of swimming capacity and energy use in seven European freshwater fish species. *Ecology of Freshwater Fish,* **17,** 284-291.

Vadstein, O., Attramadal, K.J.K., Bakke, I., Forberg, T., Olsen, Y., Verdegem, M., Giatsis, C., Skjermo, J., Aasen, I.M., Gatesoupe, F.J., Dierckens, K., Sorgeloos, P. & Bossier, P. (2018) Managing the Microbial Community of Marine Fish Larvae: A Holistic Perspective for Larviculture. *Frontiers in Microbiology,* **9**.

Varela, J.L., Ruiz-Jarabo, I., Vargas-Chacoff, L., Arijo, S., Leon-Rubio, J.M., Garcia-Millan, I., Del Rio, M.P., Morinigo, M.A. & Mancera, J.M. (2010) Dietary administration of probiotic Pdp11 promotes growth and improves stress tolerance to high stocking density in gilthead seabream *Sparus auratus*. *Aquaculture,* **309,** 265-271.

Vasconcelos, V., Azevedo, J., Silva, M. & Ramos, V. (2010) Effects of Marine Toxins on the Reproduction and Early Stages Development of Aquatic Organisms. *Marine Drugs,* **8,** 59-79.

Vazzana, M., Cammarata, M., Cooper, E.L. & Parrinello, N. (2002) Confinement stress in sea bass (*Dicentrarchus labrax*) depresses peritoneal leukocyte cytotoxicity. *Aquaculture,* **210,** 231-243.

Verner-Jeffreys, D.W., Shields, R.J. & Birkbeck, T.H. (2003) Bacterial influences on Atlantic halibut *Hippoglossus hippoglossus* yolk-sac larval survival and start-feed response. *Diseases of Aquatic Organisms,* **56,** 105-113.

Verschuere, L., Rombaut, G., Sorgeloos, P. & Verstraete, W. (2000) Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews,* **64,** 655-+.

Vine, N.G., Leukes, W.D. & Kaiser, H. (2006) Probiotics in marine larviculture. *Fems Microbiology Reviews,* **30,** 404-427.

Wedekind, C., Von Siebenthal, B. & Gingold, R. (2007) The weaker points of fish acute toxicity tests and how tests on embryos can solve some issues. *Environmental Pollution,* **148,** 385-389.

Williams, S.C.P. (2014) Gnotobiotics. *Proceedings of the National Academy of Sciences of the United States of America,* **111,** 1661-1661.

Xia, J.H., Lin, G., Fu, G.H., Wan, Z.Y., Lee, M., Wang, L., Liu, X.J. & Yue, G.H. (2014) The intestinal microbiome of fish under starvation. *Bmc Genomics,* **15**.

Yamano, K. (2005) The role of thyroid hormone in fish development with reference to aquaculture. *Jarq-Japan Agricultural Research Quarterly,* **39,** 161-168.

Zhou, Y.Z., Cattley, R.T., Cario, C.L., Bai, Q. & Burton, E.A. (2014) Quantification of larval zebrafish motor function in multiwell plates using open-source MATLAB applications. *Nature Protocols,* **9,** 1533-1548.

Zhou, X. & Wang, Y. (2012) Probiotics in Aquaculture-Benefits to the Health, Technological Applications and Safety. In: *Health and Environment in Aquaculture*, Edmir Daniel Carvalho, Gianmarco Silva David and Reinaldo J. Silva, IntechOpen.

Chapter 9 SUMMARY

Aquaculture is the fastest growing sector to produce animal protein in the world and based on the increasing human population growth, a continuously rising demand for fish through aquaculture is to be expected in the future. In addition, the capture fisheries production is stagnant and consequently, an intensification and diversification of the aquaculture sector imposes itself. In this regard, Dover Sole *(Solea solea)* is a promising aquaculture candidate as the flesh is highly appreciated in culinary circles and has a high market value.

The general introduction (**chapter 1**) of this PhD dissertation starts with an overview of Dover sole morphology, its natural occurrence, fisheries and aquaculture. Furthermore, the status and challenges in the larviculture of marine flatfish with special attention to Dover sole are discussed, hereby focusing on disease prevention as well as the research tools available. In addition, the current knowledge gaps are highlighted. In view of this, the general objective of this dissertation was to contribute to more sustainable Dover sole larviculture by developing several innovative research tools deployable to tackling some key issues hampering its development. In addition, several probiotic and prebiotic candidates were evaluated to increase Dover sole larval health and control the bacterial disease vibriosis.

Disease outbreaks are being increasingly recognized as a significant restriction to aquaculture production and large efforts are being made to combat disease. In this respect, egg disinfections are routinely applied in aquaculture facilities. However, at the initiation of the PhD research, no information on appropriate egg disinfection protocols for Dover sole was available. Therefore, in **chapter 3,** an overview of disinfection protocols in teleost eggs is given, emphasizing the most frequently used and upcoming disinfection products, the related protocols and their purposes. Furthermore, the benefits and limitations of each product are highlighted. This review helps researchers and aquaculturists to make an informed choice on egg disinfection and identifies the gaps in knowledge where future research is needed.

In this PhD research, the ultrastructural morphology of the Dover sole egg envelope from fertilization until hatching is described for the first time (**chapter 4**). These results are important for species identification and may help predict the effect of external influences on the fish embryo. In a first step, a successful protocol for scanning and transmission electron microscopy (SEM and TEM) was pinpointed. Based on both SEM and TEM, three distinct layers were determined in the egg envelope. During the development of the fish embryo, a change in the outer structure of the egg was observed. Starting from two days post fertilization, parts of the outermost layer, containing uniformly distributed pores, or two outer layers peeled off. The second deeper layer showed larger pores, with less defined edges and was more electron dense. The third innermost layer appeared to be much thicker and

possessed indentations. Prior to hatching, the compact structure of the innermost layer was distorted by dispersed holes and tears.

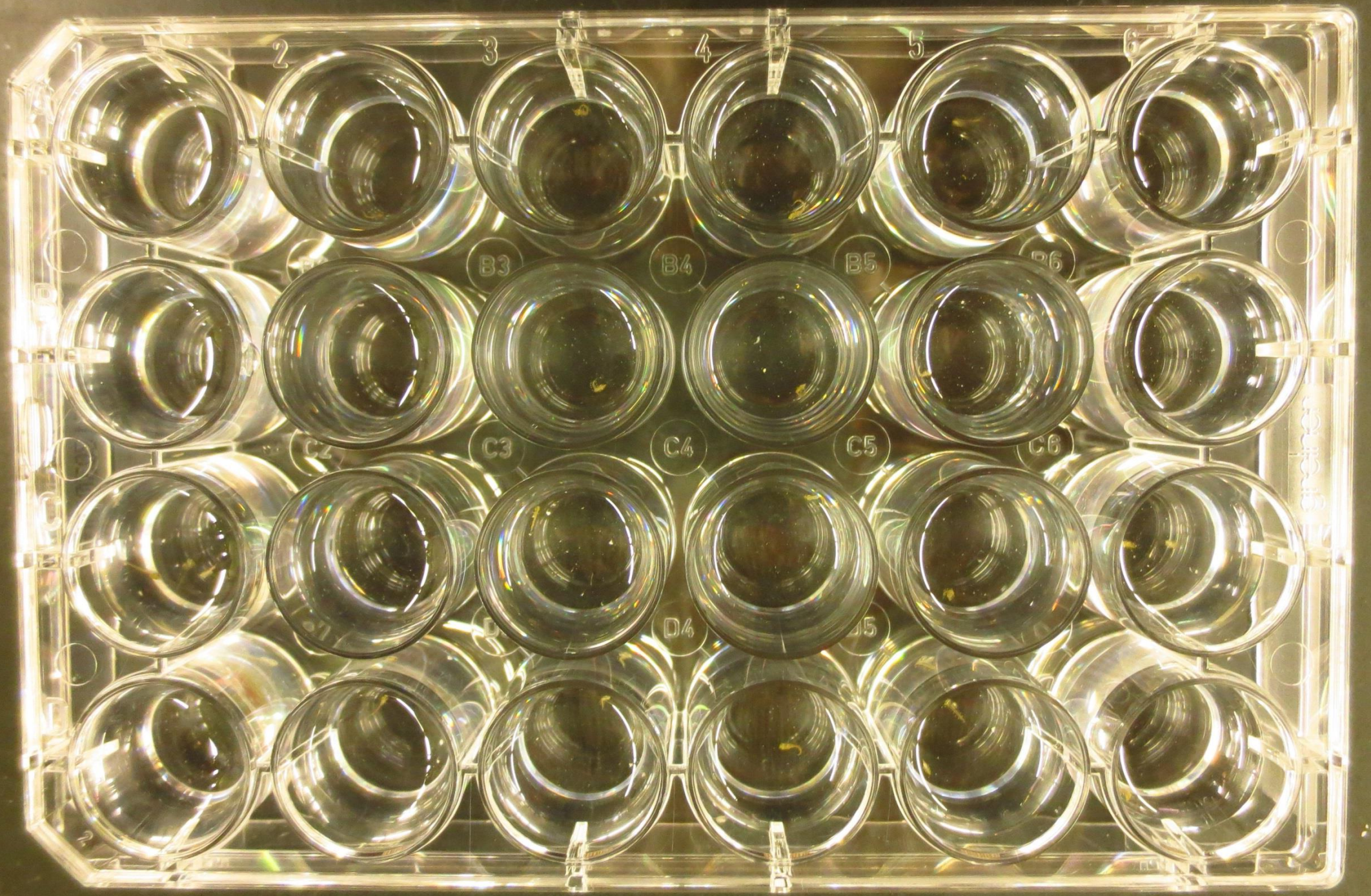
The above mentioned hindrances in Dover sole larviculture and in particular the very limited knowledge on this species, stress the need for increased research efforts on the larval production phase. A higher reproducibility as well as substantiated comparison between studies is to be expected with the use of a standardized and controlled experimental set-up. In this respect, a reliable experimental housing system was pinpointed for Dover sole larvae (**chapter 5**), whereby the eggs are disinfected with 1% hydrogen peroxide for 3 minutes and larvae are housed individually in 24-well plates. Larvae were reared until 26 days after hatching with good survival rates and normal food intake. Additionally, different disinfection products and protocols were tested with variable results on hatching and bacterial load. However, none of these consistently resulted in bacteria-free larvae enabling the development of a gnotobiotic system.

Vibriosis is a challenging bacterial disease affecting both adult and early life stages of fish with the highest incidence occurring in larval and juvenile stages of marine species. One of the main causative agents of vibriosis is *Vibrio anguillarum*, affecting more than 90 aquatic organisms including Dover sole. However, hitherto, the mode of action of this pathogen is largely unknown. To better understand the pathogenesis of vibriosis and evaluate new ways to prevent and combat this important disease, there is a great need for reliable and reproducible experimental infection models. In **chapter 6** a challenge model for vibriosis in Dover sole larvae was pinpointed. Following administration of *V. anguillarum* via immersion at 1×10^7 colony forming units/mL, a larval mortality of 50% was observed at 7 days post-inoculation (=17 days after hatching). In a next step, the applicability of the challenge model was tested and several probiotic candidates were selected to evaluate their protective potential following pathogen challenge. The probiotic potential of 371 isolates retrieved from Dover sole was assessed by screening for their inhibitory effects against *Vibrio* spp. and absence of haemolytic activity. Two remaining isolates (*V. proteolyticus* and *V. lentus*), the latter known for its protective characteristics in seabass larvae, were further tested *in vivo* by means of the pinpointed experimental infection model. Neither isolate provided via the water or feed proved to be protective for the Dover sole larvae against challenge with *V. anguillarum* WT.

In **chapter 7**, two prebiotic treatments (mannanoligosaccharide and alginic acid) were evaluated. Indeed, although immunostimulating properties are allocated to prebiotics, limited research on the potential protective effect of prebiotics against challenge with a known pathogen was performed in marine fish larvae. Although increased fish health and performance were reported for other fish species, no increased larval growth or shifted proliferation and apoptosis rate of the intestinal

epithelium were observed in Dover sole larvae following 0.2% or 0.4% mannanoligosaccharide supplementation. In contrast, larval supplementation with 0.4% alginic acid resulted in a decreased standard body length. No difference in survival was observed following administration of both compounds. For the first time, the protective potential against pathogen challenge with *V. anguillarum* following supplementation with prebiotic candidates was determined in Dover sole larvae. No increased survival was discerned following 0.2% and 0.4% mannanoligosaccharide nor 0.4% alginic acid supplementation prior to challenge. However, a decreased survival following pathogen challenge in 0.2% alginic acid supplemented larvae was observed, which might reveal a possible negative impact when administering alginic acid although further research is warranted. The latter finding highlights the importance of including both direct as well as indirect parameters to evaluate the harmlessness of new compounds.

Chapters 3 to 7 consider specific (research) studies and contain their own discussion sectors. However, the general discussion (**chapter 8**) tackles some general points in light of the combined findings hence overarching the various chapters. In addition, the advantages, limitations and opportunities to elaborate the potential of the developed models are highlighted. Furthermore, future possibilities and some reflections about ethics are mentioned.


2
3
4
5
B3
B4
B5
B6
C2
C3
C4
C5
C6
D4

Chapter 10 SAMENVATTING

Aquacultuur is de snelst groeiende sector die voorziet in dierlijke eiwitten ter wereld en door een progressieve stijging van de wereldbevolking wordt een voortdurend toenemende vraag naar vis vanuit de aquacultuur verwacht in de toekomst. Bovendien stagneert de productie vanuit de visserijsector, waardoor een intensivering en diversificatie van de aquacultuursector zich opdringen. In dit opzicht is Noordzeetong (*Solea solea*) een veelbelovende nieuwe aquacultuur kandidaat, gezien de vis sterk gewaardeerd wordt in culinaire kringen en bovendien een hoge marktwaarde heeft.

De algemene inleiding (**hoofdstuk 1**) van dit proefschrift begint met een overzicht van de morfologie van Noordzeetong, het natuurlijke voorkomen ervan, de visserij en aquacultuur. Verder worden de status en uitdagingen in de larvicultuur van mariene platvissen besproken, met speciale aandacht voor Noordzeetong. Hierbij wordt de nadruk gelegd op ziektepreventie en de beschikbare onderzoeksmodellen en worden de huidige hiaten in de kennis over de soort benadrukt. De algemene doelstelling van dit proefschrift (**hoofdstuk 2**) was dan ook om bij te dragen aan een meer duurzame Noordzeetong larvicultuur door verschillende innovatieve onderzoeksinstrumenten te ontwikkelen. Deze technieken kunnen ingezet worden om een aantal belangrijke problemen aan te pakken die het succes van de soort in de aquacultuur in de weg staan. Bovendien werden verschillende probiotische en prebiotische kandidaten geëvalueerd om de larvale gezondheid van Noordzeetong te verhogen en de bacteriële ziekte vibriose te beheersen.

Ziekteuitbraken worden steeds vaker erkend als een belangrijke beperkende factor in het succes van de aquacultuur en bijgevolg worden grote inspanningen gedaan om ziekten te bestrijden. In dit opzicht wordt desinfectie van viseieren routinematig toegepast in aquacultuurfaciliteiten. Bij de start van het doctoraatsonderzoek was echter geen informatie beschikbaar over geschikte protocollen voor het ontsmetten van Noordzeetongeieren. Daarom wordt in **hoofdstuk 3** een overzicht gegeven van desinfectieprotocollen voor eieren van teleosten, met de nadruk op de meest gebruikte en opkomende desinfectieproducten, de bijbehorende protocollen en hun doelstellingen. Bovendien worden de voordelen en beperkingen van elk product benadrukt. Deze literatuurstudie kan onderzoekers en aquacultuurexperts helpen om een gefundeerde keuze te maken omtrent ontsmetting van eieren en identificeert de hiaten in kennis waar toekomstig onderzoek nodig is.

In dit doctoraatsonderzoek wordt de ultrastructurele morfologie van de eischaal van Noordzeetong van bevruchting tot ontluiken voor de eerste keer beschreven (**hoofdstuk 4**). Deze resultaten zijn belangrijk voor de identificatie van soorten en kunnen helpen bij het voorspellen van het effect van externe invloeden op het visembryo. In een eerste stap werd een succesvol protocol voor scanning- en transmissie-elektronenmicroscopie (SEM en TEM) ontwikkeld. Op basis van zowel SEM als TEM werden drie afzonderlijke lagen in de eischaal beschreven. Tijdens de ontwikkeling van het visembryo

werd een verandering in de buitenste structuur van het ei waargenomen. Vanaf twee dagen na de bevruchting werden delen van de buitenste laag, die uniform verspreide poriën bevatten, of twee buitenste lagen afgepeld. De tweede, diepere laag vertoonde grotere poriën, met minder gedefinieerde randen en was meer elektronendens. De derde, binnenste laag oogde veel dikker en bezat indeukingen. Kort voor het ontluiken van het ei vertoonde de compacte structuur van de binnenste laag van de eischaal gaten en scheuren.

De bovengenoemde problemen in de Noodzeetong larvicultuur en in het bijzonder de zeer beperkte kennis over deze soort, benadrukken de behoefte aan een hogere onderzoeksinspanning voor de larvale productiefase. Het gebruik van een gestandaardiseerde en gecontroleerde experimentele proefopzet zou resulteren in een hogere reproduceerbaarheid evenals een beter onderbouwde vergelijking tussen studies. In dit opzicht werd een betrouwbaar experimenteel huisvestingssysteem ontwikkeld voor Noordzeetong larven (**hoofdstuk 5**). Hierbij werden de viseieren gedurende 3 minuten gedesinfecteerd met 1% waterstofperoxide. De hieruit ontloken larven werden afzonderlijk in 24-well platen gehuisvest. Larven werden aangehouden tot 26 dagen na het uitkomen met goede overlevingskansen en een normale voedselinname. Daarnaast werden verschillende desinfectieproducten en -protocollen getest met variabele resultaten op het ontluiken van de larven en bacteriële aanwezigheid. Geen van deze resulteerde echter consistent in bacterievrije larven, nodig voor de ontwikkeling van een gnotobiotisch model.

Vibriose is een uitdagende bacteriële ziekte die zowel de adulte als jonge levensstadia van vissen aantast, met de hoogste incidentie in de larvale en juveniele stadia van mariene soorten. Eén van de belangrijkste oorzaken van vibriose is *Vibrio anguillarum*, die meer dan 90 soorten van in het water levende organismen aantast, waaronder de Noorzeetong. Tot nu toe is de manier waarop deze pathogeen ziekte veroorzaakt echter grotendeels onbekend. Om de pathogenese van vibriose beter te begrijpen en nieuwe manieren te onderzoeken om deze belangrijke ziekte te voorkomen en te bestrijden, is er een grote behoefte aan betrouwbare en reproduceerbare experimentele infectiemodellen. In **hoofdstuk 6** werd een infectiemodel voor vibriose in Noordzeetong larven uitgewerkt. Na toediening van *V. anguillarum* via het water, in een concentratie van 1 x 10^7 kolonievormende eenheden/ml, werd 7 dagen na inoculatie (=17 dagen na ontluiken) een larvale mortaliteit van 50% waargenomen. In een volgende stap werd de toepasbaarheid van het infectiemodel getest en werden verschillende probiotische kandidaten geselecteerd om hun beschermend potentieel na de pathogeen toediening te evalueren. Het probiotische potentieel van 371 isolaten verkregen uit Noordzeetong werd beoordeeld door te screenen op hun inhiberende effecten tegen *Vibrio* spp. en afwezigheid van hemolytische activiteit. Twee resterende isolaten, *V. proteolyticus* en *V. lentus*, waarvan de laatste bekend is om zijn beschermende eigenschappen bij

zeebaarslarven, werden verder *in vivo* getest met behulp van het eerder ontwikkelde experimentele infectiemodel. Geen van de isolaten die via het water of de voeding werden toegediend, bleek de Noordzeetong larven te beschermen tegen besmetting met *V. anguillarum* WT.

In **hoofdstuk 7** werden twee prebiotische behandelingen (mannanoligosaccharide en alginezuur) geëvalueerd. Hoewel immuunstimulerende eigenschappen worden toegewezen aan prebiotica, werd slechts beperkt onderzoek naar het potentieel beschermend effect van prebiotica tegen infectie met een gekende pathogeen uitgevoerd in mariene vislarven. Hoewel voor andere vissoorten een betere gezondheid en prestaties werden beschreven, werd geen toename van de larvale groei of veranderde proliferatie en apoptose index van het intestinale epitheel waargenomen in de Noordzeetong larven na toevoeging van 0,2% of 0,4% mannanoligosaccharide. Daarentegen resulteerde larvale supplementatie met 0,4% alginezuur in een kortere lichaamslengte. Er werd geen verschil in overleving waargenomen na toediening van één van beide producten. Voor de eerste keer werd onderzocht of de toediening van prebiotica kandidaten bescherming bood tegen infectie met een pathogeen (*V. anguillarum*) in Noordzeetonglarven. Er werd geen betere overleving waargenomen na 0,2% en 0,4% mannanoligosaccharide noch 0,4% alginezuur toediening voorafgaand aan pathogeen infectie. Er werd echter een verminderde overleving na pathogeen toediening waargenomen in de larven gesupplementeerd met 0,2% alginezuur, wat een mogelijk negatief effect op larven bij toediening van alginezuur zou kunnen aantonen, hoewel verder onderzoek hieromtrent noodzakelijk is. Deze laatste bevinding benadrukt het belang van het insluiten van zowel directe als indirecte parameters om de onschadelijkheid van nieuwe verbindingen te evalueren.

Hoofdstukken 3 tot 7 gaan in op specifieke (onderzoeks) studies en bevatten hun eigen discussies. In de algemene discussie (**hoofdstuk 8**) worden echter enkele algemene punten behandeld in het licht van de gecombineerde bevindingen, overkoepelend over alle hoofdstukken. Bovendien worden de voordelen, beperkingen en verdere toekomstige mogelijkheden van de ontwikkelde modellen alsook enkele reflecties over ethiek besproken.

Chapter 11 DANKWOORD

Een eerste woordje van dank gaat natuurlijk uit naar mijn promotoren; **Prof. dr. Decostere**, Annemie, met het dankwoord aan jou kan ik een boek vullen. Bij mijn eerste sollicitatiegesprek bijna 7 jaar geleden dacht ik 'Die ligt me wel, daar kan ik vast goed mee samenwerken'. En gelijk had ik… Bedankt voor alles; inclusief voor je vertrouwen in mijn kunnen, zelfs als ik dat zelf even verloren had, voor alle bijgebrachte kennis en ondersteuning bij het wetenschappelijk schrijven, voor je aanwezigheid bij tegenslag. Je hebt me geleerd wat het is om een integer, open-minded onderzoeker te zijn, eerlijk en oprecht, kritisch maar ook doordacht en vooruitdenkend. Ik heb ook altijd enorm geapprecieerd hoe er in de vele vergaderingen kon gediscussieerd worden op een –zalig- open manier, waarbij we soms een (ongezouten) andere mening hadden, maar waarbij dat resulteerde in beter onderzoek, sterkere artikels, uitgebreidere meningen en dieper respect. Dit doctoraat is een gezamenlijk werk, niet alleen dankzij tekstuele verbeteringen en financiële of materiele ondersteuning, maar bovenal dankzij de mentale steun en verrijking. De afgelopen 7 jaar hebben me gevormd als onderzoeker én als mens en dat is ook grotendeels aan jou te danken. Dankjewel voor de begeleiding, maar bovenal, dankjewel voor de vriendschap.

Prof. dr. Van den Broeck, Wim, bedankt om mij in alle vertrouwen de vrijheid te geven mezelf te ontwikkelen, als doctoraatstudent en assistent. Ik heb met veel plezier de studenten warm gemaakt voor de histologie practica (geprobeerd alvast) en lichtmicroscopen hebben voor mij geen geheimen meer. Je deur stond altijd open om mijn vragen te beantwoorden.

Graag wil ik hierbij tevens de voorzitter, **Prof. dr. S. Croubels** en de leden van de examencommissie bedanken. **Prof. dr. Chiers**, Koen, bedankt om mij een stekje te gunnen op de dienst Pathologie en je hulp met de histologie. Maar vooral bedankt voor je koele, nuchtere blik op de feiten en je droge humor als 'jouw visvrouwen' grote problemen (denken) te zien. Ik zal ook altijd jouw toewijding na het kelderincident blijven appreciëren. **Prof. dr. Peter Bossier**, bedankt voor uw inbreng als ervaren jurylid in mijn examencommissie. Tevens bedankt voor uw altijd enthousiaste gesprekken tijdens meetings, symposia en congressen, en niet te vergeten uw kennis van de Gran Canarische visrestaurants. **Prof. dr. Katleen Hermans**, dankjewel voor uw input als jurylid. Tijdens onze samenwerkingen heb ik altijd enorm uw enthousiasme, eerlijkheid en engagement geapprecieerd. U bent iemand die tevens mijn interesse voor dierenwelzijn deelt. Bedankt ook voor uw spontaniteit en aanstekelijke humor. **Dr. Marie-Laure Bégout**, merci de vouloir devenir membre de mon comité d'examen et de vouloir libérer le temps nécessaire. Je tiens à vous remercier pour l'accueil chaleureux pendant mon séjour dans votre institut de recherche l'année dernière. J'étais complètement absorbée par le groupe de recherche et j'ai eu l'occasion de participer à vos activités. J' espère que nous nous reverrons à l'avenir. **Dr. Anamaria Rekecki**, hartelijk dank om mijn werk zo grondig en kritisch na te lezen en tevens te voorzien van opbouwende commentaar. Ik heb de fakkel overgenomen als assistent bij de vakgroep Morfologie

en ik ben blij dat we elkaar nu opnieuw treffen. **Dhr. Marco Dubbeldam**, uw blik vanuit de Noordzeetongkweek op grotere schaal dan het labo was bijzonder waardevol voor deze doctoraatsthesis. Tevens bedankt voor de vlotte samenwerking en aanlevering van tongeieren de laatste jaren. Ik heb ook steeds genoten van de immer enthousiaste rondleidingen doorheen jullie faciliteiten.

Prof. dr. Freddy Haesebrouck, dankuwel om mij steeds welkom te laten voelen in uw labo. Tevens bedankt voor de kritische blik en opbouwende feedback bij al mijn manuscripten alsook uw onvoorwaardelijke steun na het kelderincident.

Em. Prof. dr. Paul Simoens, mijn onderzoek ging over vissen en ik was een bioloog. In beiden was ik een buitenbeentje aan de vakgroep. Toch was u steeds enthousiast over die wondere waterwezens en heb ik mij in alles gesteund gevoeld. Dankuwel voor alle verwondering, ondersteuning en blijvend geloof in mijn kunnen. Het doctoraat is er gekomen en uw wijsheid draag ik bij me. Hopelijk kan u nog lang genieten van een rustig pensioen, met prachtige kleinkinderen en veel wondere dieren in de natuur. **Prof. dr. Cornillie**, Pieter, jouw deur stond altijd open en je was altijd bereid om te helpen. Dankjewel voor de hulp bij de masterproeven, de 3D reconstructies en de vele practica. **Prof. dr. Despiegelaere**, Ward, we hebben niet lang samengewerkt, maar zeker bedankt voor jouw open visie rond de toekomst na een doctoraat. Ook fijn dat er een andere bioloog op de vakgroep is…

De realisatie van dit werk was grotendeels mede mogelijk dankzij de input van talrijke onderzoekers met elk hun eigen expertise. **Prof. dr. L. Duchateau**, hartelijk dank voor alle statistische analyses, en het geduldig beantwoorden van al mijn vragen. De discussie rond de wellplaten blijft me intrigeren en ik heb ze dan ook niet weerhouden uit dit doctoraat. Bedankt voor uw oprechte interesse in het visonderzoek, ook al week deze experiment opbouw grondig af van de meeste proeven hier aan de faculteit. Aansluitend wil ik ook nog **Prof. dr. R. Morent** en **Prof. dr. N. Degeyter** bedanken voor de input en het gebruik en technische ondersteuning van de laser, alsook **Prof. dr. K. De Meestere** en **Lore Vandermeersch** voor het gebruik van de ozonisator en alle begeleiding hierrond. Ook dank ik **Prof. dr. N. Boon** en **dr. S. van Nevel** voor de expertise en ondersteuning inzake de flow cytometer. **Prof. dr. H. Nauwynck** en **dr. J. Van Cleemput**, bedankt voor de begeleiding met de TUNEL kleuring en de snelle en vlotte samenwerking. Verder wil ik ook mijn dank uiten aan **Prof. dr. A. Huysseune, Prof. dr. P. Witten** en **Mieke Soenens** voor de ondersteuning en begeleiding in de vele pogingen tot Epon inbedding van de tongeitjes. Ook **Prof. dr. W. Bert** wil ik bedanken voor zijn expertise. Een bijzondere dankjewel voor **Myriam Claeys**, voor jou niet aflatende enthousiasme voor de elektronenmicroscopie. Jij geloofde dat de inbedding van het tongei ging lukken, zelfs nadat ik het zelf (bijna) opgegeven had. En je had gelijk, we zijn er geraakt. **Prof. dr. Aerts**, Johan, hartelijk bedankt om me de wereld van de

cortisol en het belang van de precursoren en metabolieten te leren kennen. Stress zal nooit meer hetzelfde zijn… Dankjewel ook aan het **ILVO** en in het bijzonder **Hans Polet**, voor de immer vlotte samenwerking. Tevens bedankt aan het **VLIZ**, met in het bijzonder de **bibliotheek**, voor de omkadering en aangeleverde literatuur.

Al dit fijn en boeiend onderzoek had nooit kunnen plaatsvinden zonder, jawel, de tongeitjes. Dus zeker een bijzonder woordje van dank aan **IMARES** en in het bijzonder **Ewout Blom** om mij wegwijs te maken in de wereld van de *Solea solea* broodstock en de wispelturige tongeitjes. Daarnaast ook een dankjewel aan **Stichting Zeeschelp** met in het bijzonder **Marco Dubbeldam** voor de feilloze samenwerking en de gedeelde passie voor de vissoort.

Dit brengt me tot de collega's van de vakgroep Morfologie. Allereerst de mensen van de visbokaal, zoals mijn eerste bureau zo vaak genoemd werd. Annelies en Marlien, mijn knoflook-buddies; **Annelies**, bedankt om bij de start van dit avontuur mijn (officieuze) meter te zijn en me meer dan wie ook wegwijs te maken in het mariene onderzoek. Dankjewel voor alle ondersteuning, zowel praktisch als mentaal, die gepaard ging met de start van dit grote avontuur 'doctoraat' genaamd. Voor altijd mijn duikbuddy in Gran Canaria… Veel plezier bij het VLIZ en we komen elkaar zeker nog wel tegen… **Marlien**, de uitbundige meid met het gouden hart. Op werkvlak mijn grootste tegenpool; maar ik ben toch ook altijd wat jaloers geweest op je 'joie-de-vivre'. Zotte ritjes in je knappe vliegmachien naar Gravelines, paëlla's en dronken muzikanten in Gran Canaria, terrasjes in Las Vegas,… we vergeten ze nooit. Respect! **Maarten**, het verdwaalde testosteron in onze bureau. Gelukkig kon je wel steeds je mannetje staan tussen al dat vrouwelijk geweld en getetter. Altijd genoten van je spontaniteit, je werkijver, je humor. Ik heb nog steeds een immense bewondering voor de hoeveelheid werk die jij in 4 jaar hebt afgelegd, zonder je eigen(heid) te verliezen. **Marieke**, de rust zelve, waarmee ik de liefde voor de vislarven deelde; maar tevens de miserie van *in vivo* proeven, uitgestelde experimenten, ongeschreven artikels. We hebben er onderweg allebei wel aan getwijfeld, maar dat doctoraat en die prachtzoon hebben we beiden gehaald ;-) ! **Marjan D**, ik ben blij dat jouw carrière in de museumwereld zo goed gaat. Het is je gegund! Hopelijk hebben we jou niet teveel verveeld met onze visverhalen.

En dan zijn er nog de andere (ex)collega's, waarmee ik niet direct een bureau deel(de), maar wel een job, een passie om op deze faculteit te werken: **Kimberley en Tim**, met z'n drieën ongeveer gelijk gestart, maar allemaal met een verschillende aanpak. Ik heb genoten van onze goeie babbels en toekomstoverwegingen. Als assistenten hebben we ook vaak ons 'studenten lief en leed' kunnen delen en jullie maakten al onze practica uren samen een heel stuk fijner. Hanna en Martine, jullie vervoegden ons enkele jaren later als assistenten. **Hanna**, succes met je doctoraat, maar ik heb al gezien dat dat goedkomt, met zo een gedrevenheid en focus. **Martine**, dankjewel voor alle uren dat je me wegwijs

hebt gemaakt in de histologie, zodat ik de lessen onderbouwd kon geven. Ik wens je alle succes met je uitdagende onderzoek naar de leeftijdsbepaling bij pups. Je bent alvast heel gepassioneerd door het onderwerp. Ik ben benieuwd naar de resultaten! **Khan**, veel succes met jouw doctoraat en veel plezier met het lesgeven. **Shana**, jouw traject als assistent is nog maar pas gestart. Merci voor de leuke babbels tijdens de practica en je oprechte interesse in mensen! Jammer dat we niet langer zullen samenwerken, maar succes in wat je verder onderneemt. Ook **Charis**, alle succes gewenst bij het verdere verloop van je doctoraat.

Verder wil ik **Winny, Ymke, Monique, Sofie M, Bart VdV, Isabelle, Eva** en **Christophe** bedanken voor de vele uren dat we samen les gegeven hebben. Jullie aanwezigheid maakte de soms lange dagen des te aangenamer. Denk nog eens aan mij als jullie anatomie van de vis geven in de toekomst… ;-)

Lobke en Jurgen, bedankt voor alle hulp bij het histologisch labowerk. Jullie hebben me leren larven inbedden en gezorgd voor mooie coupes van die mini visjes, wat resulteerde in leuke 3D reconstructies. Jurgen, nog eens speciaal merci voor de vele vele vele bestellingen die je met mij geregeld hebt! Op zulke momenten: mijn held**. Liesbeth**, onze EM-specialist. Was jij er al maar eerder geweest; voor hoofdstuk 4… Maar mijn tongeitjes hebben jou bij de start alvast getoond dat het niet simpel ging worden. Geniet van Oscar! **Anja**, veel succes nog met je job en de kindjes. Misschien komen we elkaar in Denderhoutem nog eens tegen. **Bart DP**, sorry voor alle keren dat ik alweer met een dringend PC probleem in je bureau stond. Die dingen hebben me gewoon niet graag… Bedankt om me meer dan eens uit de IT nood te helpen. Bovendien staan er dankzij jou ook heel wat mooie foto's in dit doctoraat. **Patrick**, merci voor al het rijden en rotsten met vissen, voor mijn onderzoek of voor de practica en voor het klaarzetten van de tafels. De stoere brandweerman met het grote hart! Voor de administratie kon ik steeds terecht bij **Eric** en nu bij **Evelyne**. Merci voor alle geregel, alle reservaties en domme vragen van mijnentwege. Ook zeker dankjewel voor de constante voorziening aan bureaumateriaal en jawel, mijn speciale tussenbladeren ☺

Sandra, bedankt om het zo netjes te houden en af en toe eens te vragen om de bureaus op te ruimen. Wie weet start ik op mijn volgende werk toch weer met korte haren ;-)

Mijn tweede thuis aan deze faculteit bleek ik te vinden op de vakgroep Pathologie.

Prof. dr. Rik Ducatelle, hartelijk bedankt om mij op te nemen in jullie vakgroep en voor de fascinerende gesprekken rond probiotica en darmgezondheid. Ik heb ook onthouden dat wij als doctoraatsstudenten te snel vinden dat we geen/mislukte resultaten hebben maar dat net die onverwachte resultaten tot heel veel nieuwe en boeiende dingen kunnen leiden. Niks zo interessant als wat er kriebelt in onze buik en in die van de kippen, de vissen en menig ander dier.

Maaike, mijn platvis-kompaan en mede-bioloog. Ik heb genoten van al onze tijd samen. Onze chaotische start in de visstallen, de vele liters zeewater en vele dode vissen, de (winderige) zeetripjes met de Simon Stevin die mij de kans gaven nog eens 'in het veld' te staan. Ik heb je misschien mee helpen opstarten, maar man, wat een werk heb je ondertussen al verzet. Ik ben super trots op wat een sterke onderzoeker je bent. Topvrouw, nog veel succes met de laatste zware werkmaanden en vergeet daarna vooral geen vakantie te nemen ;-) Wij komen elkaar wel nog tegen, de Noordzee is zo groot niet…

Astra, the one and only office manager. Dankjewel voor je praktisch inzicht, je onuitputtelijke bron van energie (waar haal je die?), je gedrevenheid, en je probleemoplossend vermogen. Als je zegt dat je iets geregeld krijgt, dan krijg je het ook geregeld. Merci ook voor, gewoon, de leuke babbels ;-) **Delphine**, wat heb ik genoten van onze tijd samen! Altijd leuke gesprekken in het labo of tijdens de middag. Een supermama voor haar kindjes! En jij stond altijd voor me klaar met raad en daad! Dankzij jou heeft mijn zoon toch ook wat meer om aan te doen ;-) Ik zal je missen! **Christian,** jouw enorme dankbaarheid is een mooie en warme eigenschap. Merci om altijd klaar te staan voor alles en iedereen. Jij bent de rust zelve, dus daarom ook nog even: sorry voor het lawaai ☺ Ik hoop voor jou dat je nog lang kan komen werken op de dienst, tussen je collega's, je (werk)familie. **Joachim,** jouw droge humor is aanstekelijk; je hebt me vaak doen schaterlachen. Bedankt om ook altijd zo praktisch en creatief mee te denken, of het nu over onderzoek, infrastructuur of opendeurdagen ging. De 'visvrouwen' vinden jouw interesse in aquacultuur en visonderzoek uiteraard ook een groot pluspunt ;-). **Sarah**, we hebben elkaar niet zoveel gezien maar in ieder geval succes met je gezinnetje! En veel plezier met de immuno's. Moge ze allemaal slagen vanaf vandaag! **Leen,** de retrodame van de dienst, merci voor alle toffe babbels en je enthousiasme. Merci ook voor de vele verhalen uit de zaal. De een al wat smakelijker dan de andere… Ik zal ze missen. **Selina**, veel succes met jouw residentie, dat komt wel goed! **Bert en Laurien**, veel succes met jullie assistentschap en het behalen van een doctoraat. Met zoveel enthousiasme komen jullie er wel. Ik gun jullie een zaal vol interessante gevallen, maar met niet al te veel moeilijke eigenaars, zodat ze vlot afgehandeld kunnen worden! **Marjan S**, oprecht succes met het finaliseren van je doctoraat. **Norbert**, een van de weinige dierenartsen met een interesse voor de zee. Het was dan wel niet voor de vissen, maar voor de zoogdieren… en ook niet voor de levende, maar voor de dode… maar het kwam dichter in de buurt dan de meeste ;-) Jij bent onze doorzetter, de voorbeeldige student, de grappige Nederlander maar ook de grote avonturier waar we stiekem allemaal wat jaloers op zijn. Geniet van Australië, en daarna Zweden. Misschien komen we elkaar daar nog terug tegen! **Bart en Frederik**, nog niet zo lang bij de ploeg, maar wat een duo! Jullie brengen echt wel sfeer in the house en alles ligt er steeds perfect bij. Als het nòg properder wordt, kunnen we voortaan beter lunchen in de zaal ;-)

Mijn voormalige bureaugenootjes op Patho… in de eerste plaats, merci om mij direct zo welkom te laten voelen in jullie bureau. **Veronique**, wat hebben we samen veel gebabbeld! Koen kon er gelukkig nog mee lachen, met die tettergatten naast zijn deur… Maar ik heb het al gemist, dat heerlijk keuvelen, die entertainende verhalen, dat schateren tijdens de werkuren en tussendoor nog werk gedaan krijgen ook. Alhoewel ik voor het finaliseren van mijn doctoraat misschien wat meer had moeten thuiswerken dan ;-) **Leslie**, bedankt voor alle fijne gesprekken en bemoedigende woorden. Samen zwanger, samen voor de eerste keer mama, het schept wel een band. Veel plezier nog met Marilou, dat is een schatje, en succes met de zwangerschap van nummer 2.

Uit het oog maar niet uit het hart, merci voor onze tijd samen: **Els, Lonneke, Leen Claeys, Michiel, Han, Laure VDB, Jan Francies**.

Nog een kort woordje van dank aan de laboranten van Bacteriologie. In het bijzonder, **Arlette en Serge**, bedankt om mij vooral in het prille begin wegwijs te maken in het labo. En voor al die keren dat ik kwam binnengestormd met een 'probleem'. Ik apprecieer enorm de hulp die jullie altijd geboden hebben. **Serge**, merci ook voor je nuchtere kijk én droge humor. Dat helpt om te relativeren. En oja, voor die vele onverwachte autoclaven…. Vol zeewater… **Marleen**, dankjewel voor onze leuke tijd samen, voor al het geregel en om paraat te staan voor al mijn vragen. Toestellen bestellen en gebruiksaanwijzingen maken, offertes vergelijken of pakjes verzenden, het heeft geen geheimen meer voor mij. Merci om altijd zo sterk mee te leven en oprecht geïnteresseerd te zijn in de mensen. Succes nog met je pubers! Ik zal aan je denken, als die van mij zijn eerste grote kuren krijgt ;-) Merci ook aan **Gunter** voor de vele administratie, voor het samen lachen en het zoeken van het juiste materiaal. Tenslotte ook nog succes aan de (post)doctoraatstudenten van het 'kippenonderzoek': **Evy, Fien, Annatachja, Kirsten, Jill, Lore, Karen, Pearl, Venessa,…** Bedankt voor alle heerlijke lunchgesprekken, het samen klagen maar vooral het samen lachen. Till we meet again.

Ook heel wat mensen buiten het werk hebben op hun manier bijgedragen aan dit doctoraat. Hierbij een woordje aan **Frank**, de biologie en chemieleerkracht waar het allemaal mee begon. Dankjewel Frank, voor jouw liefde in wetenschap, die je zo meesterlijk hebt doorgegeven. Ik ben er door gebeten geweest. Bedankt ook voor de bezinningsdagen, de wetenschapsweek, de veilige haven die je aan ons pubers kon geven. Daarbij ook zeker een pluim voor Leen. Jullie brachten zoveel rust en wijsheid, dat mijn hart er nog steeds warm van wordt. Het was de start van dit wonderlijk verhaal. De Noordzeetong, ze zat er ook in Wimereux… naast dat lief klein konijntje… en die nacht die je alleen in films ziet…

Jasper en Annelien, bij jullie is het altijd thuiskomen. Dankjewel voor het lekkere eten (altijd en overal), het begrip en de verstrooiing, de liefde en vriendschap. Jasper, niet voor niks de peter van onze kleine vent. Wij twee, wij gaan al lang mee, zo'n 15 jaar ondertussen. We hebben het vaak gehad over mijn

werk (al dan niet bij menig tripel of trappist). Mijn grootste passie, mijn grootste geluk én verdriet. Bij deze, het is er, dat doctoraat, en met spijt in het hart wordt ook het hoofdstuk assistent afgesloten. Wie weet wat de toekomst brengt, maar ik ben er gerust op dat jullie er deel zullen van uitmaken.

De 'biologen' en hun sterke achterban, **Lies en Rutger** (en schattige Tiem), **Bart en Martijn**, **Eva en Seba**, **Elke en Hans**, **Noemi en Jonas**, merci voor alle fijne kerstdineetjes. Ook al worden onze agenda's drukker, gaan we even naar het buitenland of worden onze gezinnen groter, ieder jaar maken we toch weer tijd voor elkaar. De tafel werd al heel wat langer en binnenkort komen er nog twee stoeltjes bij… maar we blijven er voor gaan! Dankjewel om ook steevast te vragen hoe het met dat doctoraat ging en de liefde voor de biologie en de zee te delen.

Stijn en Annemie, Karen en Dimi, Katrien en Peter, Sarah en Davy, het leven is te druk en gaat te snel. De dagen zijn te gevuld, de kinderen groeien te snel en er zijn te weinig feestjes… In de ideale wereld zagen we elkaar vaker, maar als we elkaar zien, vliegt de tijd en kijk ik alweer uit naar de volgende keer.

Aan al mijn vrienden in het algemeen. Sorry voor al die dingen die ik gemist heb, alle data die ik niet vrij was, of de momenten waarop mijn hoofd niet bij jullie was. Het was de fout van de visjes ;-) Nee, eerder de fout van mijn liefde voor die visjes, mijn passie voor dit doctoraat. Maar het zit er op, dus hopelijk vinden we wat meer tijd, tussen het leven door…

Mama en papa, dankjewel voor alle kansen die jullie mij gegeven hebben. Het wordt vaak als vanzelfsprekend beschouwd maar dat is het niet. Dankjewel om altijd in mij te geloven en ook om vaak voor onze kleine Lars te zorgen. **Aurelia**, mijn *kleine* zusje, ik zal er altijd voor jou zijn. En met **Jan** heb je een ventje uit de duizend. Aan mijn schoonmoeder, **Helena**, en mijn schoonbroers en zussen, **Elger en Sarah** (en kleine Bas), **Myriam en Björn** (en Lili en Timur), **Petra**, dankjewel om mij te laten deelnemen aan jullie grote warme familie! Dankjewel ook voor de fijne weekendjes en de vele steun doorheen dit doctoraat. Hierbij mag ook een dikke dankjewel voor **Hilde en Bart, Lucie en Etienne, Stefaan en Marissa, Ann en Christophe** en **Marlies** niet ontbreken. Merci om mij op te nemen in jullie familie en ook dankjewel voor AL die lekkere taarten doorheen de jaren. Ik heb drie families, en wie heeft het geluk zoveel warmte te kennen?

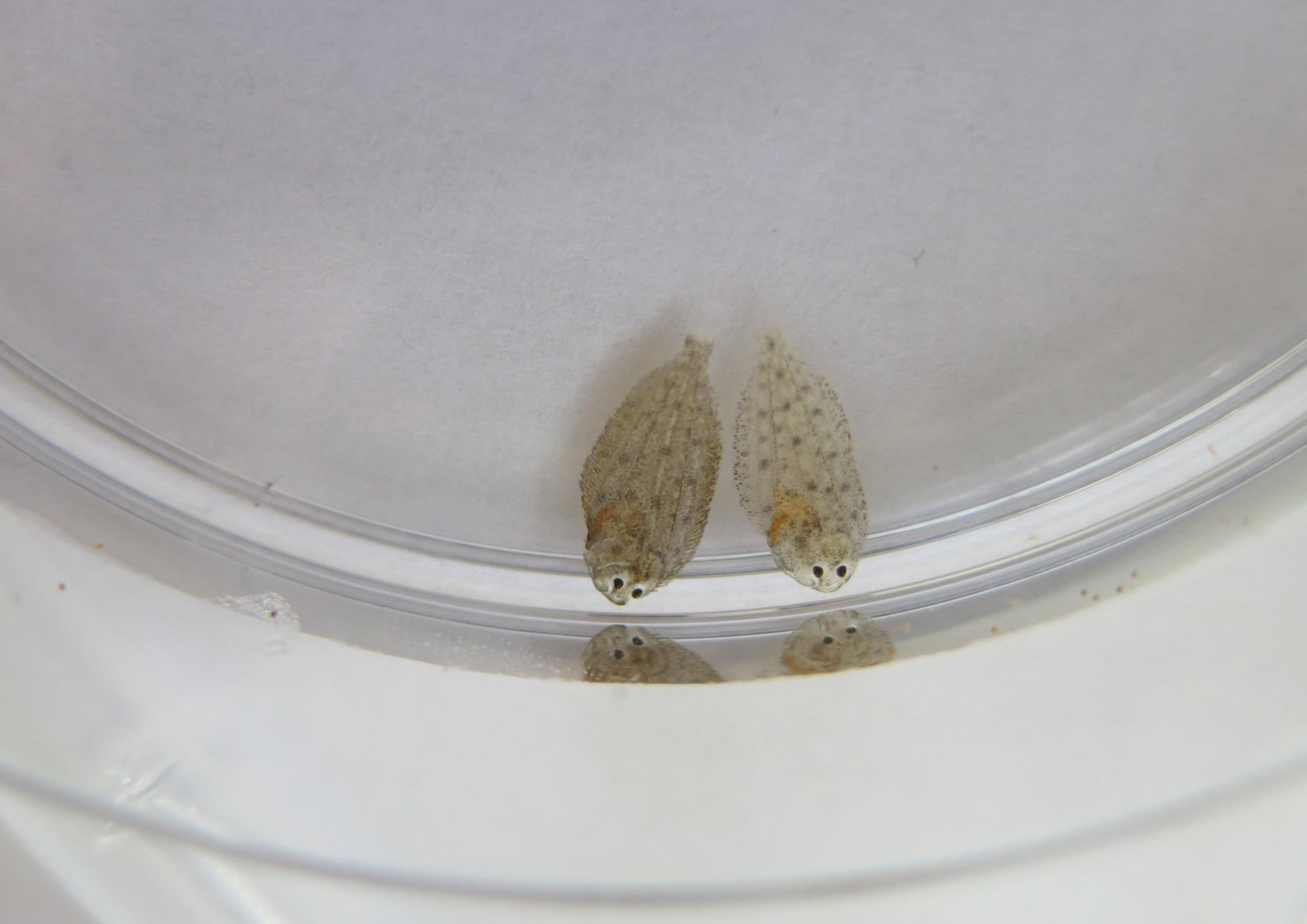
En last but definitely not least, **Diemer**, mijn egeltje, de man in mijn leven. Achter iedere sterke vrouw staat een sterkere man… Dankjewel om al meer dan 11 jaar aan mijn zijde te staan en lief en leed met me te delen. Ook de afgelopen 7 jaar, je weet wel, met die 9 to 5 job die ik gekozen had… Wij twee, dat werkt, wij werken hard en (te) veel, maar ook goed samen en voor elkaar. *Liefde is alles… ook wat er blijft als al de rest verloren is.*

Tenslotte nog **Lars** (mijn kleine vent, mijn alles, mijn mooiste prestatie): mama gaat weer wat weekends vrij zijn om te spelen ☺ . Blijf zo gedreven, blijf de wereld bewonderen, de natuur inspecteren maar bovenal, blijf eigen aan wie je bent, mijn schat. Jij hebt mama alvast leren relativeren!

Aan alle mensen die ik hier niet vermeld heb, maar die toch, op welke manier ook hebben bijgedragen aan dit doctoraat, een welgemeende dankjewel. Ook al staat dit werk op mijn naam, een doctoraat maak je nooit -echt nooit- alleen!

Liefs,

Evelien



Chapter 12 CURRICULUM VITAE

Evelien De Swaef werd geboren in Gent op 6 juni 1988. Na het vervolledigen van het secundair onderwijs, richting wetenschappen-wiskunde, startte ze in 2006 met de opleiding Biologie aan de Universiteit Gent. Ze behaalde het diploma Master in de Biologie met grote onderscheiding in 2011.

In 2012 begon ze vervolgens als assistent aan de vakgroep Morfologie van de Faculteit Diergeneeskunde, onder begeleiding van Prof. dr. Annemie Decostere en Prof. dr. Wim Van Den Broeck. Haar doctoraatsonderzoek was gericht op het bijdragen tot een duurzame Noordzeetong larvicultuur door het verkennen van verschillende factoren die het succes van de kweek beïnvloeden. Gedurende haar doctoraat werkte Evelien De Swaef nauw samen met de vakgroep Pathologie, Bacteriologie en Pluimveeziekten. Daarnaast begeleidde ze heel wat practica van verschillende vakken, met een focus op algemene en bijzondere histologie alsook de anatomie van de vis. Ze was promotor van 15 masterproeven en fungeerde als leescommissaris.

In 2014 voltooide ze aan de Universiteit Gent de opleiding ‘Master in Laboratory Animal Science’ en ze vervolledigde de assistententraining (2017) en het Doctoral School of Life Sciences and Medicine trainingsprogramma (2019) van de Universiteit Gent.

Evelien De Swaef is auteur en co-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften en trad tevens op als reviewer. Ze nam actief deel aan verschillende congressen en symposia.

Chapter 13 BIBLIOGRAPHY

13.1 SCIENTIFIC PUBLICATIONS

De Swaef, E., Vercauteren, M., Duchateau, L., Haesebrouck, F., Decostere, A. (2018) Experimental infection model for vibriosis in Dover sole (*Solea solea*) larvae as an aid in studying its pathogenesis and alternative treamtents. *Veterinary Research*, **49**, 24.

Vercauteren, M., **De Swaef, E.,** Declercq, A., Bosseler, L, Gulla, S., Balboa, S., Romalde, J.L., Devriese, L., Polet, H., Boyen, F., Chiers, K. & Decostere, A. (2018) First Isolation of *Vibrio Tapetis* and an Atypical Strain of *Aeromonas Salmonicida* from Skin Ulcerations in Common Dab (*Limanda Limanda*) in the North Sea. *Journal Of Fish Diseases,* **41**, 329–335.

Aerts, J., Schaeck, M., **De Swaef, E.**, Ampe, B. & Decostere, A. (2018) *Vibrio lentus* as a probiotic candidate lowers glucocorticoid levels in gnotobiotic sea bass larvae. *Aquaculture*, **492**, 40-45.

De Swaef, E., Claeys, M., Bert, W., Huysseune, A., Witten, P.E., Van den Broeck, W. & Decostere, A. (2017) Ultrastructural morphology of the envelope of Dover sole *Solea solea* eggs from fertilization until hatching with emphasis on sample preparation. *Micron*, **99**, 9-18. *Impact factor: 1.728, category: MICROSCOPY, rank: 4/10.*

De Swaef, E., Demeestere, K., Boon, N., Van den Broeck, W. Haesebrouck, F. & Decostere, A. (2017) Development of a reliable experimental set-up for Dover sole larvae *Solea solea* L. and exploring the possibility of implementing this housing system in a gnotobiotic model. *Research in Veterinary Science*, **115**, 418-424. *Impact factor (2017): 1.616, category: VETERINARY SCIENCES, rank: 31/140*

De Swaef, E., Van de Broeck, W., Dierckens, K. & Decostere, A. (2016) Disinfection of teleost eggs: a review. *Reviews in Aquaculture*, **8**, 321-341. *Impact factor (2016): 4.618, category: FISHERIES, rank: 2/50.*

Schaeck, M., **De Swaef, E.**, Van Den Broeck, W., Van Nevel, S., Boon, N., De Geyter, N., Morent, R., Demeestere, K., Duchateau, L., Coulombet, C., Haesebrouck, F. & Decostere, A. (2016) Germ-free sea bass Dicentrarchus labrax larval model: a valuable tool in the study of host-microbe interactions. *Diseases of Aquatic Organisms,* **117,** 177-185. *Impact factor (2016): 1.549, category: VETERINARY SCIENCES, rank: 32/136*

Van Hoestenberghe, S., Wille, M., **De Swaef, E**., Goddeeris, B. & Nevejan, N. (2015) Effect of Weaning Age and the Use of Different Sized Artemia Nauplii as First Feed for Jade Perch *Scortum Barcoo*. *Aquaculture International,* **23**, 1539–1552. *Impact factor (2015): 0.96, category: FISHERIES, rank: 33/52*

13.2 CONFERENCE CONTRIBUTIONS

13.2.1 Oral presentations

De Swaef, E., Van den Broeck, W., Haesebrouck, F. & Decostere, A. Development of the first standardized biotic challenge model for Dover sole *solea solea* (WAS Aquaculture congres, 22-26/2/2016, Las Vegas)

De Swaef, E., Vercauteren, M., Haesebrouck, F. & Decostere, A. Development of the first standardized biotic challenge model for Dover Sole (*Solea solea*) and its validation by assessing the protective potential of probiotic candidates (VLIZ Marine Science Day 2017, 03/03/2017, Brugge)

De Swaef, E., Vercauteren, M., Haesebrouck, F. & Decostere, A. The key issue to larval health research in Dover sole *Solea solea* L.: a reliable experimental set-up and challenge model, as exemplified by assessing the protective potential of probiotic candidates (11th International Flatfish symposium, 11-17/11/2017, Saint-Malo, Frankrijk)

13.2.2 Poster presentations

De Swaef, E., Schaeck, M., Van Den Broeck, W., Bossier, P, Dierckens, K. & Decostere, A. Gnotobiotic models for seabass (*Dicentrarchus labrax*) and Dover sole (*Solea solea*): The chain is only as strong as its weakest link (Aquaculture 2013: Celebrating 40 years of Aquaculture, 3-7/11/2013, Las Palmas, Gran Canaria)

De Swaef, E., Schaeck, M., Van Den Broeck, W., Bossier, P., Dierckens, K. & Decostere, A. Gnotobiotic models for seabass (*Dicentrarchus labrax*) and Dover sole (*Solea solea*): The chain is only as strong as its weakest link (VLIZ Jongerencontactdag Mariene Wetenschappen 2013, 07/3/2014, Brugge, Belgium)

De Swaef, E., Claeys, M., Bert, W., Huysseune, A., Witten, P.E., Van den Broeck, W. & Decostere, A. Electron microscopic characterization of fertilised dover sole *Solea solea* eggs during embryonic development (11th International Flatfish symposium, 11-17/11/2017, Saint-Malo, Frankrijk)

13.3 SCIENCE EVENTS

Dag van de Wetenschap: stand op wetenschapsbeurs: 'Probiotica bij Noordzeetong larven' (22/11/2015, Ghent, Belgium)

Dag van de Wetenschap: stand op wetenschapsbeurs: 'Alle kleine visjes worden groot… of niet?'(27/11/2016, Ghent, Belgium)