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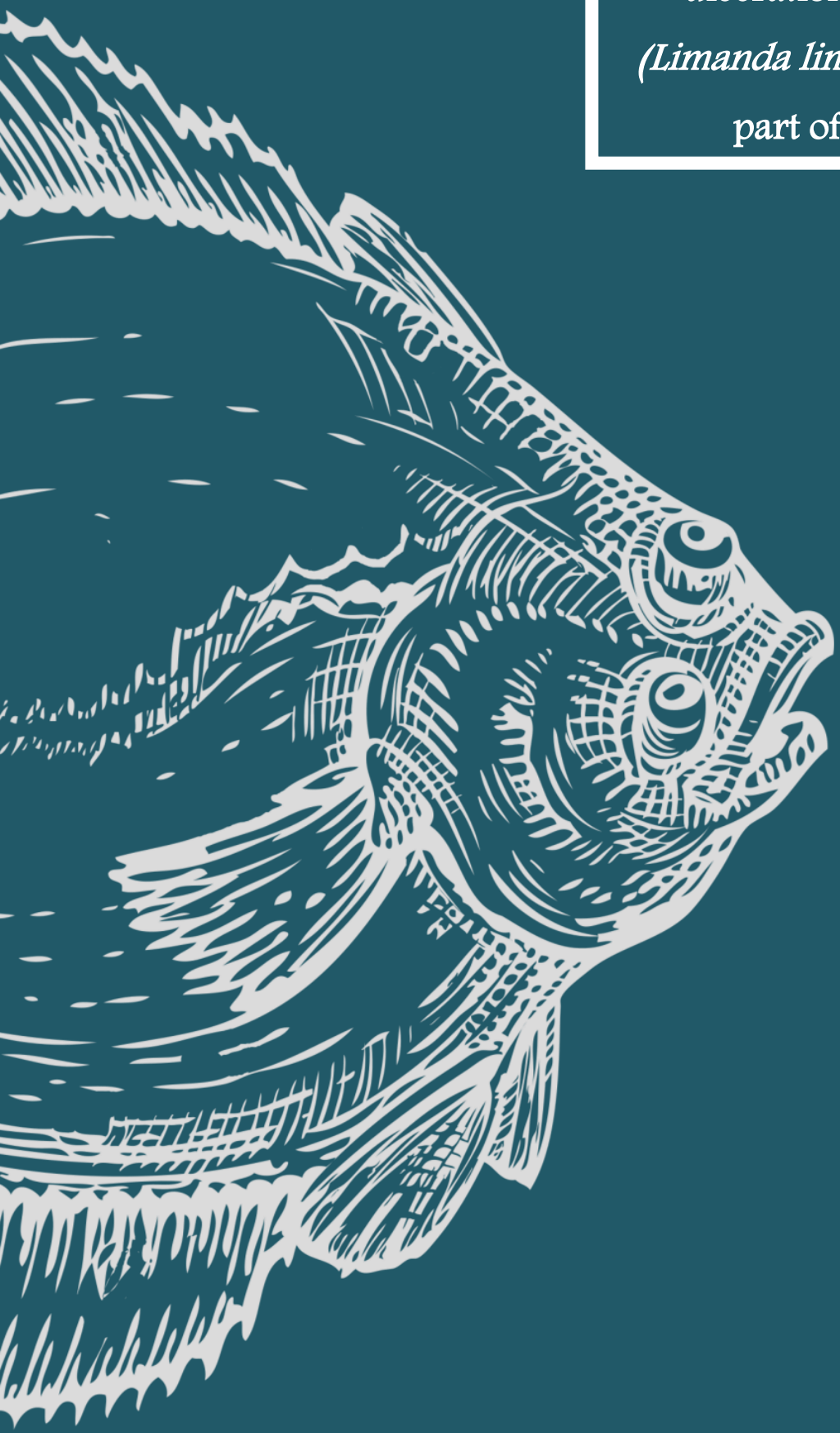

Maaike Vercauteren

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Unravelling the etiology of skin ulcerations in common dab *(Limanda limanda)* in the Belgian part of the North Sea

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

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The most asymmetrically shaped and behaviorally

lateralized of all the vertebrates, the flatfishes,

are an endless source of fascination

to all fortunate enough to study them.

Alexander M. Schreiber

Abbreviations

AIC	Automatic Identification system
AMO	Atlantic Multidecadal Oscillation
BAC	Background Assessment Criteria
BNS	Belgian part of the North Sea
BRD	Brown ring disease
C	Completely healed ulceration
CFU	Colony forming units
CI	Confidence interval
CO_2	Carbon dioxide
CONT	Control sample or group
CORT	Cortisol-fed group
CPUE	Catch per unit effort
CT	Chemical treatment
CTD	Conductivity-Temperature-Depth probe
D	Fish that died during the experimental period
DAISE	Delivering Alien Invasive Species Inventory for Europe
DMEM	Dulbecco's Modified Eagle's Medium
DPI	Days post inoculation
EAC	Environmental Assessment Criteria
EGC	Eosinophilic granulocyte
EMODnet	European Marine Observation and Data Network
EXPL	Skin explant
F	Female fish
FCS	Fetal calf serum
FDI	Fish Disease Index
FSSW	Filtered Sterilized Sea Water
GC	Goblet cell
GLM	General linear model
GLMER	General linear mixed-effect model
GUS	Gross ulceration score
H&E	Haematoxylin and Eosin staining
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPF	High power field
HPI	Hypothalamic-pituitary-interrenal axis
HPLC	High performance liquid chromatography
HSC	Hypothalamic-sympathetic-chromaffin cell
ICES	International Council for Exploration of the Sea
ILVO	Flanders Research Institute for Agriculture, Fisheries and Food
K	Fulton body condition factor (With K_B and K_E the condition at the beginning or end of the experiment)

L	Length (With L_B and L_E the length at the beginning or end of the experiment)
M	Male fish
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MHC	Major histocompatibility complex
ML	Maximum likelihood
MLSA	Multilocus sequence analysis
MS-222	Tricaine Methanosulfonate
MSFD	Marine Strategy Framework Directive
MSO	Marine Station Ostend
MT	Mechanical treatment
NP	Non-pigmented side of the fish
NT	Non-treatment
P	Pigmented side of the fish
PAS	Periodic Acid Schiff staining
PBS	Phosphate-buffered saline
PCB	Polychloorbifenyl
PCC	Plasma cortisol concentration
PCNA	Proliferating cell nuclear antigen
PLA	Polylactic acid
PSU	Practical salinity unit
RAMP	Reflex action mortality predictors
RAPD-PCR	Randomly Amplified Polymorphic DNA PCR
RBC	Red blood cell
RMS	Red Mark Syndrome
RV	Research vessel
S	Fish that survived until 21 DPI
SCC	Scale cortisol concentration
SDG	Sustainable Development Goals
SPF	Specific pathogen free
T4SS	Type IV secretion system
TA	Total affected area
TCBS	Thiosulfate citrate bile salt sucrose agar
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TUS	Tag Ulceration Score
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
U	Ulceration
UA	Ulcerative area
UPLC- MS/MS	Ultra-performance liquid chromatography coupled to tandem mass spectrometry
UV	Ultraviolet
VLIZ	Flanders Marine Research Institute
VMS	Vessel Monitoring System
W	Weight (With W_B and W_E the weight at the beginning or end of the experiment)
WBC	White blood cell
WGPDMO	Working Group on Pathology and Diseases of Marine Organisms

Chapter 1

General introduction

1.1 The marine ecosystem

The extensive marine ecosystem consists of oceans, estuaries, shorelines, tide-pools, barrier islands and salt marshes (Palumbi *et al.*, 2009; NOAA, 2018). The ocean covers more than 70 % of the earth's surface, forming a continuous body of salty water (NOAA, 2018). This ecosystem houses an immense diversity of marine life ranging from single-cell organisms to gigantic whales. Besides being a diverse system, it is also highly connected, as approximately all life depends on the existence of others. An example is the various interconnected food webs that are present in the oceans (Nunez, 2019).

The marine ecosystem is important for life on earth. Phytoplankton, mostly single celled photosynthetic organisms floating in the water column, can absorb carbon dioxide (CO_2) to synthesize carbohydrates and during this process they release oxygen, just like plants. They produce about half of the world's oxygen. Furthermore, since oceans cover over 70% of the earth's surface, they absorb a major portion of the thermal energy radiated by the sun. The ocean currents distribute this heat around the world and help regulate our climate (WWF, 2018; Nunez, 2019).

The functionality of the ecosystem depends mainly on its diversity and the balance between all of its life forms and processes (MEA, 2005; Palumbi *et al.*, 2009). The marine ecosystem, just like any other ecosystem, can have an optimal or healthy state. In 2013, Tett and coworkers defined ecosystem health as: '*a condition of a system that is self-maintaining, vigorous, resilient to externally imposed pressures, and able to sustain services to humans. It contains healthy organisms and populations, and adequate functional diversity and functional response diversity. All expected trophic levels are present and well interconnected, and there is a good spatial connectivity amongst subsystems.'*

1.2 Fascinating inhabitants of the marine ecosystem

Flatfish (Order Pleuronectiformes) are interesting creatures living in this extensive marine ecosystem. They form an order of bony fish (Class Actinopterygii, Infraclass Teleostei) consisting of approximately 822 species divided in 14 families. Flatfish inhabit marine, estuarine and, to a lesser extent, freshwater habitats; ranging from cold to tropical regions (Munroe, 2015). Adult fish may range in size from a few centimeters to over two meters in length (e.g. Atlantic halibut (*Hippoglossus hippoglossus*)) (Schreiber, 2013; Munroe, 2015). They can be recognized by their flattened body with both eyes located on the same side of the head (eye side) and associated lateralized swimming posture (Figure 1.1). Flatfish are the only known vertebrates with such a remarkable asymmetry (Schreiber, 2006; Schreiber, 2013). The eye side, also called pigmented side due to its brownish color, faces the water column and the blind or non-pigmented side is typically white and faces the sediment (Venizelos & Benetti, 1999; Bolker and Hill, 2000).

Figure 1.1: Example of the flattened body and extra-orbital position of the eyes of common dab (Limanda limanda).

With their flattened body, flatfish are perfectly adapted to a benthic lifestyle, living in or on top of the seabed. Besides the shape of their body, they possess other adaptations that help them thrive in their habitat. First, they have the ability to perfectly adjust their pigmented side to the color and even the (spotted) pattern of the sediment surrounding them (Burton, 2010). This background matching is based on visual stimuli followed by nervous or hormonal responses. These responses can change the number of pigment cells or cause an intracellular rearrangement of pigment-containing granules inside the pigment cells or melanophores. The granules can be aggregated in the center of the cell or dispersed into the dendritic extensions causing the fish to appear paler or darker, respectively (Svensson & Sköld, 2011). Besides playing a role in camouflage or cryptic coloration, pigmentation provides protection against ultraviolet (UV)-radiation, assists in thermoregulation and can play a role in inter- and intra-species communication (Svensson & Sköld, 2011).
Secondly, flatfish can burry themselves in the sediment by vigorous beats of the head in combination with undulating movements of their body (Gibson *et al.*, 2015). To allow visual inspection of the surroundings whilst being buried, the eyes have an extra-orbital position so that they remain above the sediment (Figure 1.1). This is believed to be an adaptation to the burying behavior (Gibson *et al.*, 2015). Both adaptations play an important role in the avoidance of predators (Burton, 2010).

1.2.1 The economic value of flatfish

Flatfish are not only fascinating; they are also gastronomically appreciated and therefore commercially valuable for the fishing industry worldwide. As an example, both Atlantic and Pacific halibut (*Hippoglossus stenolepsis;* Dutch common name: “heilbot”) are important economic flatfish species in Northern Atlantic and Pacific Ocean (FAO, 2019a; Fishbase, 2019). In terms of landing

volume and market value, Dover sole (*Solea solea;* Dutch common name: "tong") and plaice (*Pleuronectes platessa*; Dutch common name: "pladijs" or "schol") are economically the most important flatfish species for the Belgian fishing industry (Velghe & Scherrens, 2019).

Dover sole (Family Soleidae) is an oval, brownish flatfish living in sandy and muddy sediments with a black spot on the pectoral fin (Fishbase, 2019). They have a wide geographic distribution and can be found in the Eastern Atlantic and Mediterranean Sea, even southwards in Senegal (FAO, 2019a). Dover sole is an important economic species with worldwide reported captures of 32,057 tonnes in 2016 (FAO, 2019a). Between January and July of 2019, in total 1,310 tonnes Dover sole was landed by Belgian fishermen, with a total value of approximately € 16 million (€ 12.22 per kg) (Bellefroid *et al.*, 2019).
Plaice belongs to the family Pleuronectidae, living in sandy and muddy sediments in marine and brackish environments (Fishbase, 2019). They can be recognized by their brownish color on the eye side with round red or orange spots irregularly distributed over the body (FAO, 2019a). This species is present in the Northern Seas from the European coast to the White Sea including areas around Iceland (FAO, 2019a). Plaice is the most important flatfish for fisheries in Europe with up to 116,699 tonnes caught worldwide in 2016 (FAO, 2019a). Between January and July of 2019, 1,939 tonnes of plaice was caught and landed by the Belgian fishermen with a total value of approximately € 4.3 million (€ 2.22 per kg) (Bellefroid *et al.*, 2019).
Both sole and plaice are mainly caught using the classic beam trawl technique whereby a net provided with tickler chains is dragged over the seabed collecting flatfish as well as other benthic organisms including shrimp (*Crangon* spp.) and common starfish (*Asterias rubens*) (= bycatch) (Soetaert *et al.*, 2015). Despite concerns about the negative impact of this technique (e.g. large bycatches, high fuel costs and large impact on the seabed) it is the most frequently used technique in the Belgian fleet (Velghe & Scherrens, 2019).

Besides the above described commercially important flatfish, other species can be found in the Belgian part of the North Sea (BNS) such as common dab (*Limanda limanda*; Dutch common name: "schar"), flounder (*Platichthys flesus*; Dutch common name: "bot"), turbot (*Scophthlamus maximus*; Dutch common name: "tarbot") and brill (*Scophthalmus rhombus*; Dutch common name: "griet") (FAO, 2019a). These species are in most cases encountered as bycatches of fisheries targeting sole and plaice, sometimes resulting in high discard rates (WGNSSK, 2015).

1.3. Changing ocean and its consequences

1.3.1 Changes

The marine ecosystem is a delicate system, considerably impacted by human activities, which are increasingly diversifying (e.g. fisheries, deep-sea mining, marine biotechnology, offshore wind). These activities affect nearly all parts of the oceans and threaten the functionality and health of the marine ecosystem (MEA, 2005; Nunez, 2019). Well-known anthropogenic impacts are overfishing, habitat destruction and pollution (WWF, 2018). Recently, climate change was added to this list when the intimate relation between climate change and the oceans was acknowledged for the first time in the Paris Agreement adopted in 2015 by the UN Framework Convention on Climate Change (Lescrauwaet *et al.*, 2018). The main effects of climate change on the ocean are related to the increasing

temperature (+ 0.85 °C) and related emission of greenhouse gasses such as CO_2 (+ 127 ppm) in the atmosphere (NOAA, 2018; WMO, 2018). Further increases are predicted, with variable severity depending on protective actions that are undertaken in the following years (IPCC, 2018).

The decreasing health of the marine ecosystems is not only a conservation issue; the oceans also provide various socio-economic important ecosystem services for humans. They supply food, receive and assimilate waste, provide a livelihood for millions of people and generate tourism income by providing recreational opportunities (Beaumont *et al.*, 2007; WWF, 2018). Many of these ecosystem services depend on the biodiversity of the marine ecosystem and its connections with other systems (MEA, 2005; Palumbi *et al.*, 2009). As the ecosystem is under pressure, so are its services that humanity relies on. For example, with one degree increase in sea water temperature, an estimated loss of three million tonnes catch potential is expected (WWF, 2018). Furthermore, effects of climate change can cause more extreme weather events and rising of the sea level (WWF, 2018).

1.3.2 Consequences

Human activities at sea can result in patterns of human-induced changes in marine ecosystems which need to be understood better to achieve a sustainable management of our seas and oceans. A particular challenge is to gain knowledge of cause-effect chains, whereby human activities are considered the cause, and the effects are the impacts on local or global scale. As is frequently the case, a lack of knowledge is existing on the exact effects of anthropogenic driven changes in the marine ecosystem. Nevertheless, far-reaching consequences are expected affecting ecosystem health and functioning (Stocker, 2015). The highly connected nature of ecosystems can result in a complex and profound effect even with relatively small disturbances. For example, overfishing can result in collapse of the fish stock. However, since fish are the important predators, a more broad-range impact on lower trophic levels can be expected (Scheffer *et al.*, 2005). Both habitat destruction and pollution are known to cause structural and functional changes in various marine communities (Islam & Tanaka, 2004). A recent 'hot topic' in the area of anthropogenic pollution, is the plastic pollution in aquatic systems. In 2014, it was estimated that 5.25 trillion plastic particles were present in the sea affecting nearly 700 species of different sizes (Xanthos & Walker, 2017). Plastics can cause entanglement leading to starvation, suffocation, wounding and mortality. Moreover, plastic particles can be ingested therefore leading to a blockage of the digestive system (Baulch & Perry, 2014). Also micro-plastics (plastics < 5 mm), often associated with contaminants, are commonly mistaken by many fish species for plankton, therefore ingested and thus increasing the exposure of fish to these contaminants (Xanthos & Walker, 2017). Aquatic pollution is also regularly linked to increases in diseases prevalence (Lang, 2002; Islam & Tanaka, 2004; Stentiford *et al.*, 2009; Vethaak *et al.*, 2009; Vethaak, 2013; Lang *et al.*, 2017). Common diseases that have been associated with pollution are fin rot/erosions, skin ulcerations, skeletal deformities and liver tumors (Lang & Dethlefsen, 1996; Lang & Wosniok, 2008; Vethaak, 2013).

Climate change also has a great impact on the marine ecosystem. The impact of increased absorption of thermal energy in the oceans can have far-reaching effects, ranging from changes in worldwide currents to changes in water chemistry such as salinity fluctuations and changes in nutrient distributions related to increased stratification (Stocker, 2015; WWF, 2018). Temperature is also very important for biological processes. Growth rate and overall performance of fishes can be linked to an optimal temperature resulting in a range shift of species. Many biological processes such as spawning and migration are also linked to an environmental temperature cue (Bolle *et al.*, 1994; Gibson, 1997;

Pimentel *et al.*, 2014). Increasing prevalence of diseases can be linked to changing temperatures in the oceans, although the exact effects are still difficult to predict. The link between sea water temperature increase and changing disease prevalence has already been proven for coral pathogens (Kushmaro *et al.*, 1998), oyster disease outbreaks (Cook *et al.*, 1998) and mortality of gorgonians and other epi-benthic organisms in 1999 (Cerrano *et al.*, 2002). Baker-Austin and co-workers (2013) have provided empirical evidence that the emergence of *Vibrio* spp. diseases is linked to sea surface temperatures. Secondary effects of climate change and its linked temperature increase can also be expected as reported by Hayes and colleagues (2001). They have described the drought in the Sahel region of Africa as an effect of climate change. This drought increased the amount of iron-rich dust, which subsequently enriched marine ecosystem causing a shift in available micronutrients and consequently changes pathogen growth and virulence (Hayes *et al.*, 2001). We should be concerned about the plausible increases in disease prevalence since diseases can on their turn have a profound effect on the ecosystem by reducing the reproduction, reducing fish stocks and therefore reducing biodiversity of the marine ecosystem.

Next to the warming of the oceans, another mentioned issue is the absorption of atmospheric CO_2 in the ocean (up to 30 % of the emission). This changes the seawater chemistry and results in a drop (estimated between 0.4 and 0.5 units) in pH level, termed "ocean acidification" (Pimentel *et al.*, 2014; WWF, 2018). This ocean acidification was mainly expected to affect calcifying organisms such as mollusks and other bivalves that depend on a stable pH level to build their calcium-based shells (WWF, 2018). However, other species can also be influenced. For instance, in Senegalese sole (*S. senegalensis*), the increased temperature and ocean acidification resulted in a reduction in hatching success and larval survival and an increased prevalence of skeletal deformities due to defective skeletogenesis (Pimentel *et al.*, 2014).

1.4 Integrated marine policy

Many uncertainties remain regarding the increasing anthropogenic influence on the marine ecosystem. For example, the link between anthropogenic or environmental stressors and the development of diseases is fiercely discussed. Nevertheless, the exact etiology has in most cases not yet been pinpointed. One of the priorities, as stated by Harvell *et al.* (2002), "*to predict the anthropogenic impact is the introduction of monitoring programs to assess the prevalence and impact of several diseases in a wider range of natural systems*".

The need for monitoring was regulated in an appropriate policy. In the European Union (EU), the Marine Strategy Framework Directive (MSFD) was put in place to protect the marine ecosystem (2008/56/EC). The MSFD can be considered as the environmental pillar of the EU Integrated Maritime Policy and provides a common framework to establish environmental targets for the protection and conservation of the marine environment (Lescrauwaet *et al.*, 2018). With the research projects within MSFD, a contribution is made to the Sustainable Development Goals (SDG) formulated by the UN General Assembly (UNGA), mainly with regard to SDG14 aiming to "conserve and sustainably use the oceans, seas and marine resources for sustainable development" (Lescrauwaet *et al.*, 2018). The MSFD aims to achieve a good environmental status (GES) by 2020. This GES is roughly similar to the definition of a healthy ecosystem, as mentioned before, describing a clean, healthy and productive ocean (Lyons *et al.*, 2010; Borja *et al.*, 2013). To accomplish this, 11

quality descriptors, 29 associated criteria and 56 indicators were formulated, including subjects regarding biological diversity, fisheries and contaminants. The MSFD was translated to a Belgian royal decree on June 23, 2010, whereby concrete 'environmental targets' were formulated.

Descriptor 8 of the MSFD states that "*contaminants must not be detected at concentrations that give rise to pollution effects*". When interpreting this descriptor using an 'ecosystem approach', focusing on the effects on the entire ecosystem rather than on specific species or populations, pollution effects should be considered at various levels of biological organization. One of the environmental targets (Target 39) of this descriptor defined for the BNS is related to the control of fish diseases using mandatory integrated fish diseases monitoring (KRMS, 2008). In this case, fish diseases represent a biological effect defined as "the response of an organism, population, or community to changes in its environment" (Lyons *et al.*, 2010). Therefore, fish diseases can indicate a link between contaminant exposure or environmental changes and ecological end-points. It detects the impact of substances rather than their presence. An advantage of using this technique instead of a direct measurement of the contaminant is that it indicates an effect and therefore, the effect of substances that would not be analyzed in normal routines can still be monitored (Lyons *et al.*, 2010).

1.5 Fish disease monitoring

The purpose of fish disease monitoring is to gather information on the disease prevalence and thus the health of fish populations on a regular basis (Pettijohn, 1983). Based on the previously demonstrated link between development of diseases and environmental stressors (Lang, 2002; Vethaak *et al.*, 2009; Lang *et al.*, 2017), data gathered during monitoring campaigns can be used as proxy for the health status of the ecosystem. It also offers opportunities to monitor changes, both in short and long term and investigate spatial and temporal variability to find possible correlation with factors involved in the pathology (Pettijohn, 1983; Lang, 2002). This highlights the importance of fish disease monitoring. As mentioned before, the monitoring of fish diseases in Belgium is mandatory in the context of the environmental targets pinpointed in the Royal Decree (June 23, 2010) linked with the MSFD (KRMS, 2008), and was executed since the mid '80s in the BNS in the framework of the national environmental impact assessments of the International Council for the Exploration of the Sea (ICES).

1.5.2 Main fish diseases

Besides the liver pathologies, the FDI focusses on nine externally visible diseases of which five key skin diseases, one gill disease and three parasites (Lang & Wosniok, 2008). Skin diseases are of particular importance as biomarker for stressful environments due to the delicate nature of the skin and its intimate contact with the aquatic environment, rich in pollutants and pathogens (Noga, 2000).

An overview of the externally visible skin and gill diseases, their disease-specific weighting factor and presumed etiology is provided in Table 1.1. The most common diseases are lymphocystis and epidermal papilloma, although recent increases in hyperpigmentation were observed (WGPDMO, 2019). Based on the weighting factor, indicating the estimated importance for the well-being of the host, X-cell gill disease (9.00) has the highest impact on the host followed by fin rot/erosion (6.01) and skin ulcerations (2.12) (Lang & Wosniok, 2008). Many questions on the etiology of the lesions remain unanswered, probably due to the complex and multifactorial cause of various diseases.

1.5.1 Monitoring techniques

The first reports on fish diseases were made around 1885 (McIntosh-lymphocystis) and most of the diseases known hitherto were already described in the beginning of 1900 (Mellergaard & Nielsen, 1995). However, it was only in 1960 that the above mentioned association between fish diseases and the presence of environmental stressors, mainly pollution, became clear (Lang, 2002), resulting in an increased awareness on the importance of general and standardized monitoring.

ICES has been involved in the initiation of the general monitoring efforts and has developed basic guidelines for standardized monitoring of 13 fish diseases (Bucke *et al.*, 1996). This standardization enables comparison and combination of different monitoring efforts in the North Sea and adjacent areas such as the Irish Sea and the western Baltic Sea (Lang & Wosniok, 2008). Since 1980, monitoring has been conducted on a regular basis in some ICES member states, mainly in Germany, the UK and the Netherlands (Lang *et al.*, 2017). This standardized monitoring became part of the recommended techniques by the Oslo and Paris Commission (OSPAR) for general and contaminant-specific biological effect monitoring under the Joint Assessment and Monitoring Program (Lang *et al.*, 2017).

The methodologies are currently still evolving and recently a more holistic approach for fish disease monitoring was developed based on a Fish Disease Index (FDI) (Lang & Wosniok, 2008). This is a numeric, easy-to-interpret index implying an overall disease status of the fish. The index is based on nine externally visible diseases, two macroscopic liver neoplasms and five histologically-visible liver pathology's (Lang & Wosniok, 2008). The presence/absence of each disease is multiplied by the severity grade and by the disease-specific weighting factor. Adjustments are made for confounding factors such as size of the fish, sex and sampling season (Lang & Wosniok, 2008). The scores of each disease are summed and results in the final FDI for individual fish (a score between 0 and 100) with low values indicating healthy fish and high values diseased fish. The mean FDI of a population can be calculated from the individual FDI and the assessment of the sample FDI score can be based on established Background Assessment Criteria (BAC) and Environmental Assessment Criteria (EAC) as demonstrated by Lang *et al.* (2017).

1.5.3 Bio-indicator species used in monitoring

Common dab has been used as a sentinel or bio-indicator species in many biological effect monitoring programs (Vethaak *et al.*, 2009; Lang *et al.*, 2017) and the international ICES fish disease monitoring (Bucke *et al.* 1996). It is a benthic organism and therefore lives close to the sediment where exposure to pollution is likely to be high (Vethaak, 2013). This species has a broad geographic distribution and is the most abundant flatfish species in the North Sea (Daan *et al.*, 1990; Bucke *et al.*, 1996; WGNSSK, 2015). Moreover, common dab is sensitive to the development of various diseases (Bucke *et al.*, 1996). All these factors combined aid in the use of common dab as sentinel species (Bucke *et al.*, 1996). In section 1.6, information on the biology of this species is discussed. In the Baltic Sea and coastal or estuarine areas, flounder is more suitable due to limited abundance of common dab in these regions (Bucke *et al.*, 1996; Vethaak *et al.*, 2009).

Table 1.1: Overview of the five key skin diseases and the key gill disease that are used in the international fish disease monitoring for flatfish with the diseases specific weighting factor used in the Fish Disease Index between brackets. A higher weighting factor indicates a higher estimated impact on the host. A short description of the disease is provided and the knowledge on the etiology.

Disease	Description	Etiology	Reference
X-Cell gill disease (9.00)	Thin and pale fish, opercula slightly raised, creamy white and swollen gill lamellae; lesions are likely to result in mortality	Parasite *Xcellia lamelliphila*	Feist & Bass, 2017
Fin rot or erosion (6.01)	Thickening of epidermis in fins, subsequent necrosis of soft tissue and exposure of fin rays	Unknown, bacterial involvement (*Aeromonas* spp. *Pseudomonas* spp. and *Vibrio* spp.)	Bucke *et al.*, 1996
Skin ulceration (2.12)	Rounded, hemorrhagic lesions with white peripheries.	Unknown, bacterial involvement	Wiklund, 1994; Mellergaard & Nielsen, 1997
Lymphocystis (1.99)	White to reddish nodules(2 mm)	Iridovirus	Hick *et al.*, 2016
Hyperpigmentation (1.99)	Dark greenish colorations on the pigmented side and white areas on the non-pigmented side	Unknown	Venizelos & Benetti, 1999; Lang *et al.* 2015
Epidermal papilloma (1.98)	Smooth, opaque, slightly raised, creamy white to pink lesions	Possibly viral	Mellergaard & Nielsen, 1997

1.5.4 Benefits and controversies of the monitoring

Fish diseases are part of natural processes. This substantiates the importance of long-term monitoring programs focusing on the frequency of a diseases and (sudden) changes in this frequency, instead of presence-absence of diseases (Noga, 2000). Based on the data of long-term surveys, differentiation between natural variation of disease prevalence and (human caused) relevant changes is possible (Noga, 2000).

The hitherto used monitoring technique is merely based on a semi-quantitative scoring of the presence of diseases with addition of intensity and estimated impact on the host when using the FDI (Lang & Wosniok, 2008). Although, this is sufficient for gathering crude information and monitor changes in prevalence, in many cases, it has proven to be insufficient to provide information on the

actual cause of diseases. The reports on monitoring data and their results have been heavily discussed since only a few cases have provided undebatable evidence of a causal link between pollution and increased prevalence of diseases (Lang & Dethlefsen, 1996; Lang, 2002). Most studies can only show a correlation between environmental factors and disease prevalence (Lang, 2002).

The complexity of the ecosystem, its interactions and a plausibly multifactorial etiology of diseases will probably obscure causal relations (Lang, 2002). Furthermore, a temporal separation between the initiation of the disease and the observation impedes studying direct causal links (Noga, 2000). Another complicating factor is the limited repertoire of responses of the tissue and cells to disruption, therefore, different causal factors can have similar results in terms of histopathological changes. This again increases the complexity to establish a well-defined cause-effect relationship (Noga, 2000; Law, 2001).

To overcome these controversies, an integrated, multidisciplinary research approach, as aimed for in the present PhD study, is necessary to identify possible causative factors of certain pathologies (Lang, 2002).

1.5.5 Results of monitoring in the Belgian part of the North Sea

As part of the MSFD, the Flanders Research Institute for Agriculture, Fisheries and Food (ILVO) has been performing bi-yearly fish disease monitoring since 1985 in the BNS (Devriese *et al.*, 2015). In the spring of 2011, a sudden increase in prevalence of skin ulcerations was observed, with maximal prevalence observed in the autumn of 2013 (up to 7 %) (Figure 1.2) (Devriese *et al.*, 2015). This observation was supported by reports of fishermen (for dab and sole). Together with the increase of skin ulceration prevalence in the BNS, a similar increase was observed in the German Bight with increases from 1.1 % to 2.0 % in summer and 0.4 % to 2.6 % in winter (WGPDMO, 2012). Moreover, in the Polish economic exclusive zone of the Baltic Sea, an increase in skin ulcerations in cod (*Gadus* spp.) was observed in 2011 with prevalence reaching 4.4 % (WGPDMO, 2012).

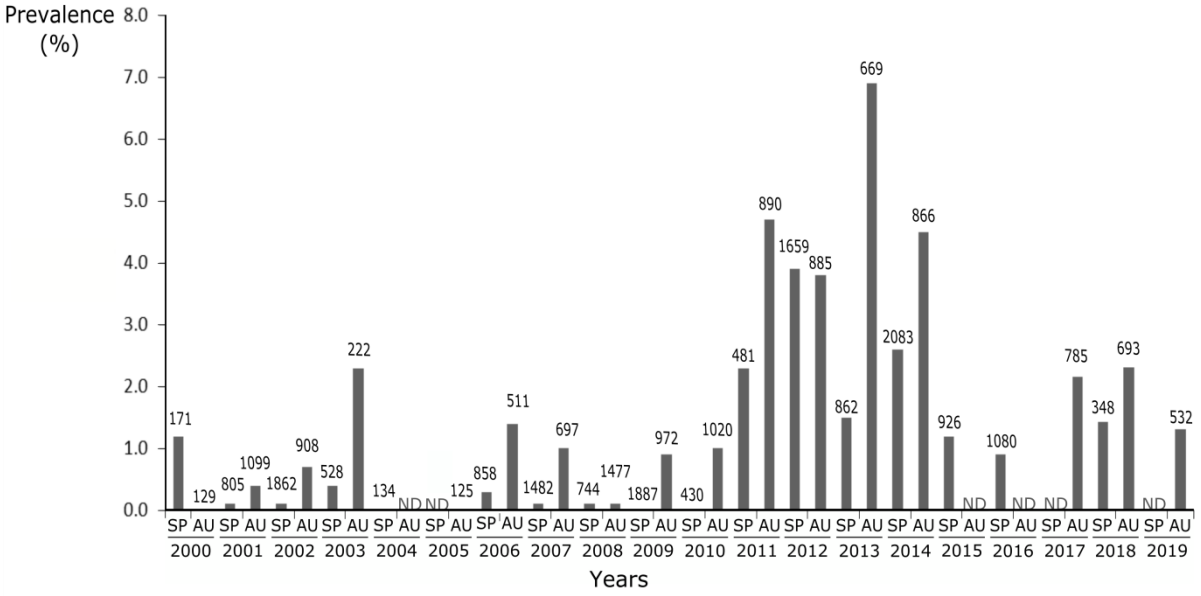

Figure 1.2: Evolution of average disease prevalence (calculated as the number of fish with skin ulcerations divided by the total number of fish caught) of skin ulcerations in common dab in the Belgian part of the North Sea. Data collected during the standardized two-yearly (spring: SP; autumn: AU) monitoring survey by the Flanders Research Institute for Agriculture, Fisheries and Food (ILVO) with RV Belgica. The numbers above each bar represent the number of fish caught ND: Prevalence not determined; survey was canceled.

This sudden increase in skin ulceration prevalence preoccupied the scientific and sea-guarding communities since no explanation for the observed increase could be provided (Devriese *et al.*, 2015), forming the base for this PhD research.

1.6 Skin ulcerations

Ulcerations are skin lesions, therefore, to study these lesions, one should know the normal morphology of the skin. The skin is an important organ for fish since it functions in communication, sensory perception, locomotion, respiration, excretion, thermal regulation and helps in maintaining osmotic homeostasis (Rakers *et al.*, 2010; Elliott, 2011b). Moreover, it acts as an important physical and immunological barrier between fish and its aquatic environment, containing more harmful chemical substances and pathogens compared to the aerial alternative (Noga, 2000; Rakers *et al.*, 2010).

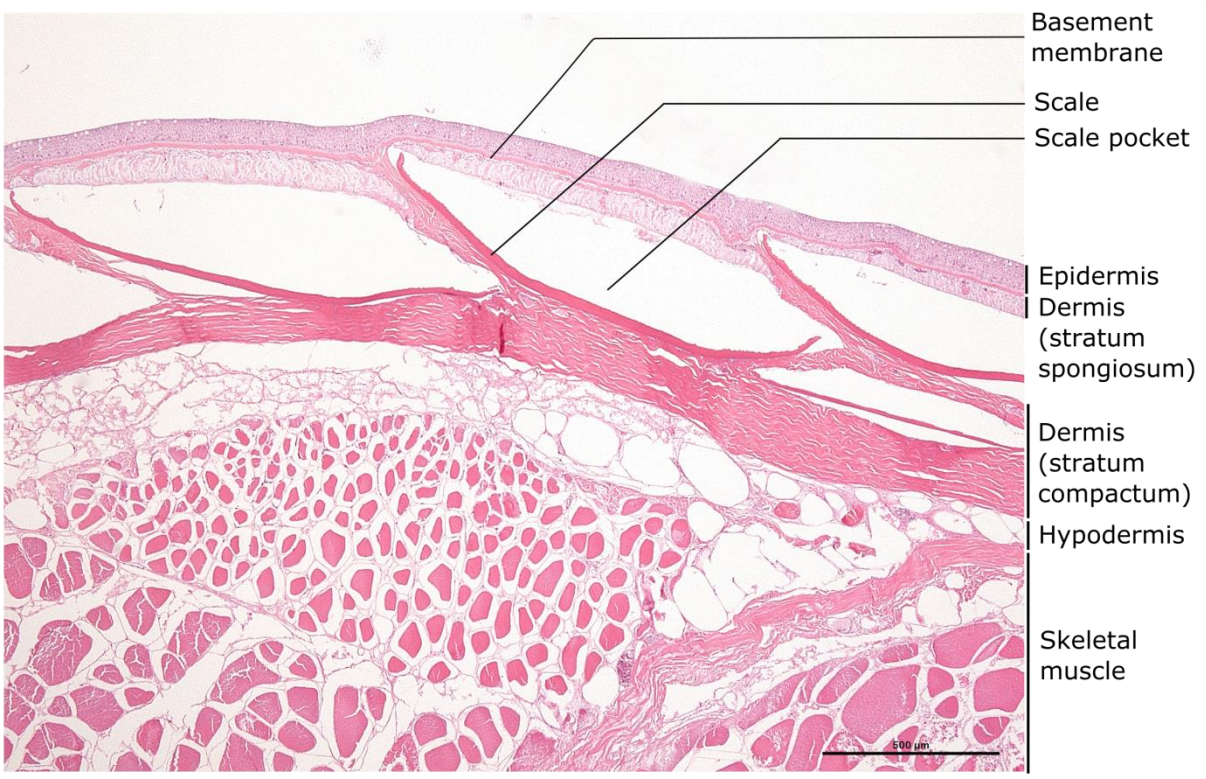

Figure 1.3: Histological view of the skin of fish demonstrating the different layers present.

As in other vertebrates, the skin of fish consists of an outer epidermal and an inner dermal layer, separated by a basement membrane (Rakers *et al.*, 2010; Elliott, 2011a). The epidermis is a multilayered structure mainly containing epithelial cells (Figure 1.3). It is metabolically active in all layers and can respond quickly to stressors (Iger *et al.*, 1995). Goblet cells, unicellular exocrine glands producing the mucus layer or cuticle, differentiate from epithelial cells in lower layers of the epidermis (Figure 1.3). When reaching the surface, the cell membrane will rupture; the cell will release its content after which it will die (Elliott, 2011a). By continuous production and release of mucus, a "dynamic mucus coat" (Svendsen & Bøgwald, 1997) is created that assists in protecting the fish against pathogen invasion by mechanical removal of the pathogens. Presumably, it can function

similarly when pollutants are present. Furthermore, the excreted mucus contains various antimicrobial substances aiding in protection such as lectines, proteolytic enzymes and other antimicrobial peptides and proteins (Elliott, 2011b). Beneath the epidermis, the dermis can be found, containing fibrous connective tissue, subdivided in two main layers, stratum compactum and stratum spongiosum (Figure 1.3). Next to fibrous connective tissue, it contains other specialized dermal elements such as chromatophores and scales. Furthermore, it contains blood vessels, nerves and adipose tissue (Elliott, 2011a).

Due to its important barrier function, any impact causing a breach in this skin barrier can be expected to have far-reaching effects on the health of the fish (Noga, 2000). Skin ulcerations (Figure 1.4) are an example of such lesions; they are defined as lesions where both epidermis and basement membrane are absent, leaving the dermal tissue exposed, often associated with inflammatory responses. From a microscopic perspective, an event (e.g. contact with toxin) can cause cell injury in the epithelial cells. In case this injury is irreversible, the cell undergoes a process resulting in cell death, causing necrosis of the epithelial tissue. Such focal necrotic zones can be sloughed together with the basement membrane causing ulcerations (Law, 2001).

On a macroscopic scale, three stages are distinguished (Bucke *et al.*, 1996). First, the acute stage of skin ulcerations is described as round, open, hemorrhagic lesions with exposed muscle tissue and a white border (Wiklund, 1994; Bucke *et al.*, 1996). Second, healing ulcerations can be recognized by reduced hemorrhages, with formation of scar tissue. The third stage, healed ulceration, shows complete closure of the lesion forming a scar (Bucke *et al.*, 1996). Importantly, skin ulcerations should be differentiated from a wound, the latter being caused purely by a direct traumatic, physical damage such as a wound caused by fishing gear (Davis & Ottmar, 2006).

The first report of skin ulcerations was made in 1905 with a description of this disease in flounder (Johnstone, 1905). The author assumed that the lesion resulted from bites of lamprey (Petromyzontiformes), presumably because of the round shape. Since then, skin ulcerations are commonly observed in various species ranging from whiting (*Merlangius merlangus*) (Devriese *et al.*, 2015) to sea cucumber (*Apostichopus japonicus)* (Deng *et al.*, 2009). However, flatfish seem to be the most vulnerable to the development of skin ulcerations (Wiklund & Bylund, 1993).

1.6.1 Prevalence

Based on the monitoring data gathered in the context of the international ICES monitoring, various reports have been published regarding skin ulceration prevalence in common dab (Table 1.2). Skin ulceration prevalence generally ranges between 0 and 10 % with reported seasonal and temporal variations. In the report of the ICES Working Group on Pathology and Diseases of Marine Organisms (WGPDMO) of 2017, a general decreasing trend in skin ulceration prevalence was described (WGPDMO, 2018). This was confirmed by Vethaak (2013) who reported a decreasing trend in skin lesions of flounder for the Dutch Wadden Sea over the last 15 years. The best example of this decrease is the prevalence of skin ulcerations on Dogger Bank. This region was always one of the hotspots of skin ulcerations, with prevalence up to 22 % in 2001. Since 2008, a decrease in ulceration prevalence was noted with contemporary prevalence of 1.3 % (Lang *et al.*, 2017; WGPDMO, 2018).

Although a general decrease is observed, some sudden increases in prevalence as in the BNS do occur. Mellergaard and Nielsen (1997) also reported a sudden increase in skin ulcerations in the Skagerrak region above Norway. The prevalence rose from approximately 0.5 to 4.4 % in 1989. In 1991, the prevalence had again lowered to 1 %; this increase was hypothesized to be related to an increased fishing intensity (Mellergaard & Nielsen, 1997).

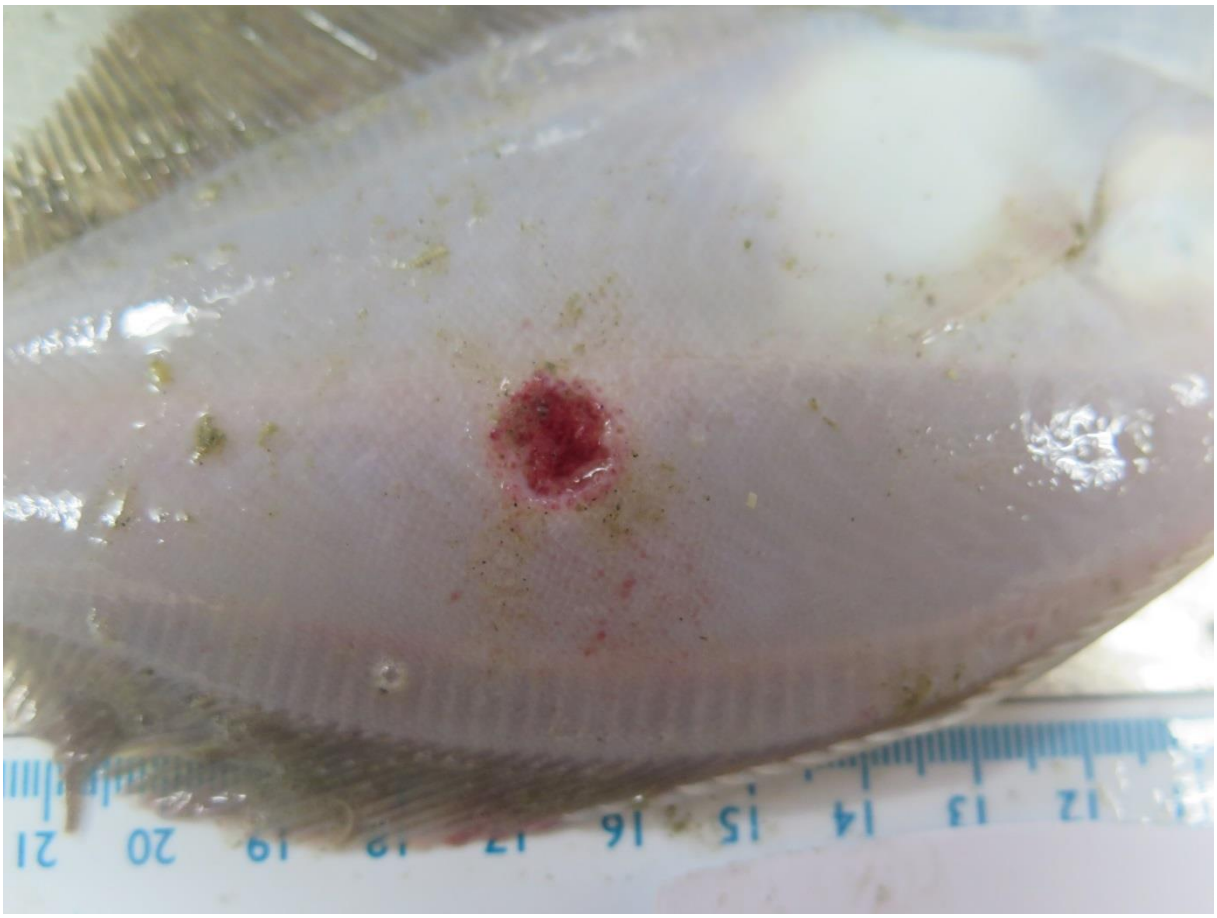

Figure 1.4: Example of an acute stage of a skin ulceration on the non-pigmented side of a common dab (Limanda limanda).

1.6.2 Impact of skin ulcerations

The impact of skin ulcerations on the fish is not yet fully understood. Skin ulcerations may result in a loss of appetite (Vilar *et al.*, 2012), change in swimming behavior (Vilar *et al.*, 2012), loss of reflexes (Davis & Ottmar, 2006), decreased condition of the fish (Mellergaard & Nielsen, 1997, Vethaak et al., 2011) and even death (Mellergaard & Nielsen, 1997; Vethaak *et al*., 1992; Vethaak, 2013). The latter might be associated with the inability to maintain the osmotic balance due to a breach in the skin barrier (Mellergaard & Nielsen, 1997; Vilar *et al.*, 2012). Noga (2000) reported that a lesion damaging 10 % of the body surface could result in mortality.

Again, these lesions are not only important from an ecological point of view. Fish with skin ulcerations lose their commercial value and are aesthetically displeasing, which can negatively affect the already declining fishing industry (Noga, 2000).

1.6.3 Etiology of the skin ulcerations

Despite their economic and ecological importance and despite the great effort, the exact cause of these lesions remains unraveled. Various reasons can be listed on why this lack of knowledge on the etiology of skin ulcerations still exists. In this case, "complexity is key". Both fish and the marine environment are complex systems, they have an intimate relation, and a multifactorial cause of these lesions can be suspected (Lang *et al.*, 1999; Stentiford *et al.*, 2009). The complex marine environment is literally a sea of pathogens, polluting agents and other disturbances, and fish live in intimate contact with this environment (Rakers *et al.*, 2010). Therefore, small changes can have large-scale effects resulting in ulceration development. This adds some complexity to understanding the primary cause. Furthermore, as mentioned above, the temporal separation between the development of lesions and the detection is a major issue in studying all wildlife diseases, including skin ulcerations (Noga, 2000; Law 2001). Skin ulcerations can thus be compared to a "cold crime scene", where the crime happened a (long) time ago and only the results remain (Law, 2001).

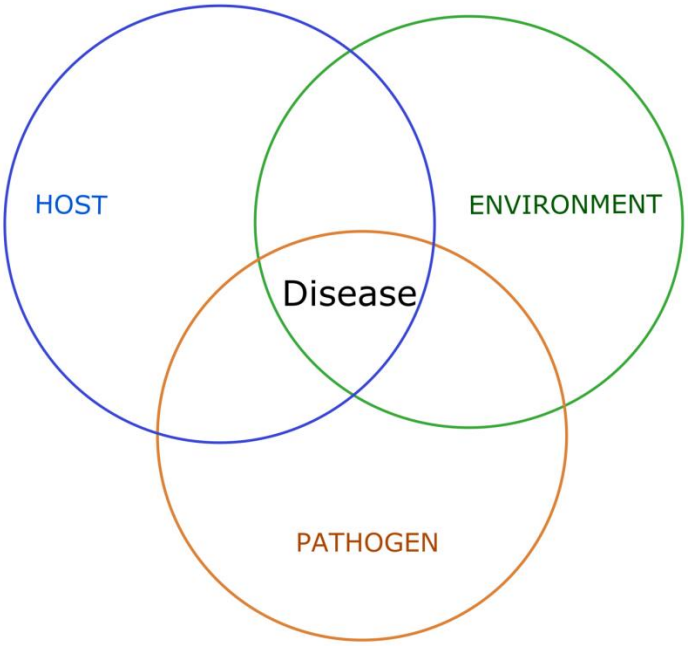

Figure 1.5: The epidemiological triad as proposed by Snieszko (1974).

A general framework for understanding these complex etiologies of infectious diseases was provided by Snieszko (1974). He proposed a model with three interdependent factors - host, pathogen and environment - which ultimately can result in the development of an infectious disease (Figure 1.5). Snieszko (1974) stated that "an *overt infectious disease occurs when a susceptible host is exposed to a virulent pathogen under proper environmental conditions*". Thus, changes in prevalence of a disease are likely linked to an imbalance between (all) these three factors (Wiklund, 1994).

1.6.3.1 Pathogen

The skin is a living tissue and therefore a common target for the large number of pathogenic microorganisms present in the sea. These pathogens can quickly colonize open wounds (secondary infection) impeding the identification of an initiating cause of skin ulcerations (Law, 2001). To find a direct link between a disease and a pathogen, experimental studies are necessary (Noga, 2000). Nevertheless, various microorganisms have been identified that potentially can cause skin ulcerations or colonize open wounds, including fungal species (Vogelbein *et al.*, 2001), viruses (Jensen & Larsen, 1982), parasites (Law, 2001) and bacteria (López *et al.*, 2011), with the latter forming the majority (Wiklund *et al.*, 1999). Various bacterial species such as *Mycobacterium ulcerans* (Eddyani *et al.*, 2004), *Tenacibaculum maritimum* (Vilar *et al.*, 2012)*, Vibrio* spp. (Colwell & Grimes, 1984), and *Aeromonas* spp. (Wiklund, 1990; Magnadottir *et al.*, 2002) have been associated with ulcerative diseases in wild fish.

Besides this direct effect of pathogens, an indirect effect can also be observed (Noga, 2000). An example is the production of biotoxins by the dinoflagellate *Pfiesteria* causing epidermal edema and necrosis and subsequent ulceration development in Atlantic Menhaden (*Brevoortia tyrannus*) (Noga, 2000; Law, 2001; Vogelbein *et al.*, 2001).

1.6.3.2 Host

Differences in susceptibility, both within and between species, stress the importance of the host in the development of skin ulcerations. Fish-related or endogenous factors that can be important in this matter are, among others, sex, immunologic capacity and the thickness and intactness of the epidermal tissue (Wiklund & Bylund, 1993; Mellergaard & Nielsen, 1997; Law, 2001; Vethaak *et al.*, 2009).

Sex is frequently pointed out in literature as a predisposing factor for skin ulcerations. However, contrasting results on the susceptibility of females and males were noted (Wiklund & Bylund, 1993; Mellergaard & Nielsen, 1997; Lang *et al.*, 1999).

The thickness of the epidermal tissue can depend on various fish-related characteristics such as different locations on the body, sex, size of the fish, condition, and degree of sexual maturation (Elliott, 2011a). In flatfish, the potential difference between the eye and blind side must also be taken into account. This asymmetry is fiercely discussed, and both sides are macroscopically and presumably microscopically distinct. Furthermore, hormones such as prolactin can also affect the thickness of the epidermis due to their role in cell proliferation (Noga, 2000). Thickness of the epidermal tissue is important since it is correlated with the susceptibility to skin damage (Elliott, 2011a).

Evidently, previous trauma of the skin can be a major contributing factor in the development of skin ulcerations. However, since many opportunistic pathogens are present in the aquatic environment, they can quickly colonize open wounds, therefore stressing the intricacy of distinguishing the proximate (infectious agents) from the ultimate causes (specific stressor leading to the wound, directly or indirectly) (Noga, 2000).

Finally, an interrelated and therefore important plausibly predisposing factor is stress. The concept of stress in fish is a frequently discussed subject. Stress can be broadly defined as “a state of threatened homeostasis” (Barton, 2002). Stress is a result caused by biotic or abiotic challenges or stressors (i.e.

stressful stimuli). When experiencing a stressor from its environment, this will elicit an activation of the hypothalamus-pituitary-interrenal (HPI) axis, equivalent of the brain-pituitary-adrenal axis in terrestrial vertebrates (Wendelaar Bonga, 1997; Barton, 2002). The HPI axis activation will result in a production of glucocorticoids in the interrenal cells in the kidney of the fish (Pankhurst, 2011). Cortisol is the principal corticosteroid hormone in Actinopterygii fishes (Barton, 2002). This stress-hormone will be released in the bloodstream and transported to various organs. Circulating cortisol concentration, especially during long-term or chronic increases, can have various physiological effects on cell and tissue (secondary effect) and population level (tertiary effects) (Barton, 2002). One of the detrimental effects is the suppression of the immune system therefore increasing the susceptibility to ulceration development (Noga, 2000). Moreover, cortisol is reported to induce stress-related changes in the skin of fish with reported increased apoptosis in epidermal and mucous cells (Iger *et al.*, 1995).

1.6.3.3 Environment

Aquatic environments are highly complex ecosystems that are being impacted in many ways. Both natural and anthropogenic exogenous or environmental factors can have an impact on the development of skin ulcerations. Many interrelated pathways and stressors can lead to dramatic epidermal injury and cell death (Noga, 2000; Law, 2001).

First, natural environmental factors such as water temperature (Wiklund & Bylund, 1993; Mellergaard & Nielsen, 1997) and salinity fluctuation (Mellergaard & Nielsen, 1997; Vethaak, 2013) have been proven to have a direct or indirect impact on the development of skin ulcerations (Noga, 2000). The study of Mellergaard and Nielsen (1997) could, albeit not significantly, show some correlations between ulceration development and oxygen-deficiency. In brown trout (*Salmo trutta*), high or low pH levels caused epidermal damages (Segner *et al.*, 1988). Changes in water quality might cause cell injury that can result in necrosis and the development of skin ulcerations (direct impact). However, the same changes can cause an activation of the stress axis resulting in a higher susceptibility of the fish for infections and therefore a higher risk for ulceration development (indirect) (Law, 2001). Importantly, the environment can have an effect on both fish and pathogen. An example is global warming. As a result, it is suspected that the effective range of a pathogen can be increased due to larger areas with the optimal growth temperature for this microorganism (Harvell *et al.*, 2002). At the same time, the susceptibility of the fish, linked to the immunogenic capacity, can also change with regard to the temperature. This can not only increase the risk for disease outbreaks but can also cause an introduction of a new pathogen in an environment with fish living in suboptimal temperatures, suffering from immune suppression.

Secondly, anthropogenic factors are definitely important. A number of studies have associated skin damage with various chemical toxicants or stressors, ranging from municipal sewage, copper, polycyclic aromatic hydrocarbons to algal toxins in the environment (overview provided by Noga, 2000). Polluted waste discharge was suggested causing increased ulceration prevalence in winter flounder (*Pleuronectes americanus*) (Khan, 2006). This pollution can cause an increased stress response in fish resulting in immunosuppression, but it can also cause damage to the skin and hereby creating an opportunity for pathogens to colonize these wounds and cause skin ulceration (Noga, 2000).

Another important anthropogenic factor with possible influence on skin ulceration development is fishing. Various studies have linked increased fishing intensity to skin ulcerations (Möller 1981; Mellergaard & Nielsen 1997). Vethaak (1992) could, however, not substantiate this hypothesis. Common dab is a typical bycatch species, therefore, they are commonly discarded which might introduce injured fish with lesions in the environment (Lüdemann, 1993; WGPDMO, 2012). Mellergaard & Nielsen (1997) hypothesized that, since ulcerations were merely observed in fish between 18 and 23 cm, that those fish had higher changes of escaping the nets of commercial trawls or being discarded. This might introduce more injured fish in the environment (Mellergaard & Nielsen, 1997). Lüdemann (1993) also found a correlation between fishery-induced skin injury and the length of the fish.

Since 2011, a new alternative electric pulse fishing technique has been used as part of an experimental trial period. The technique is used in greater North Sea including the BNS. In this technique, the mechanical stimulation caused by the tickler chains or bobbins in the beam trawls, is replaced by an electric stimulation using electric pulses. As Soetaert *et al.* (2015) summarized, this adaptation results in reduced contact with the sediment, more selectivity and therefore less discards and lower fuel costs. Previous research studies did not succeed to induce the development of skin ulcerations after exposure to the electric shocks in sole and cod (Desender *et al.*, 2016; Soetaert *et al.*, 2016). Therefore, so far, no experimental proof exists for the link between pulse fishing and the increase of skin ulceration prevalence. This pulse fishing technique should be taken into account when studying the cause of skin ulcerations since the prevalence increase in the BNS in 2011 coincided with the introduction of this pulse fishing (Devriese *et al.*, 2015).

1.6.3.4 Isolated effect?

Understanding the events that could lead to disease in fish is quite complex. It is unlikely that only one stressor acts at a time. Possibly various stressors can interact and cause an additive or synergistic effect ultimately increasing the sensitivity of the fish (Noga, 2000; Snieszko, 1974). Furthermore, each causative agent can act through various direct or indirect pathways (Law, 2001). A grasp of complex interactions that might be involved during skin ulceration development are depicted in Figure 1.6. At the beginning of the PhD study, information on the exact role of all three factors or their interactions in the development of skin ulcerations in common dab, generally and in specific case of the BNS, was meagre.

Furthermore, it must be taken into account that a dose-dependent response continuum might be at play. This can mean that depending on the concentration of a certain stressor, severity of the developing lesion or its impact on the fish survival can change. As an example, if the concentration of a certain pollutant is high enough, the fish will probably die acutely due to toxic effects of the pollutant. If lower levels are present, fish may survive longer but will develop clinical signs such as skin ulcerations. Depending on the fish, it can either overcome this lesion development and recover, or die from the effects of this lesion. If even lower amounts of pollutants are present, it is possible that no skin ulcerations or other symptoms are evident (Noga, 2000).

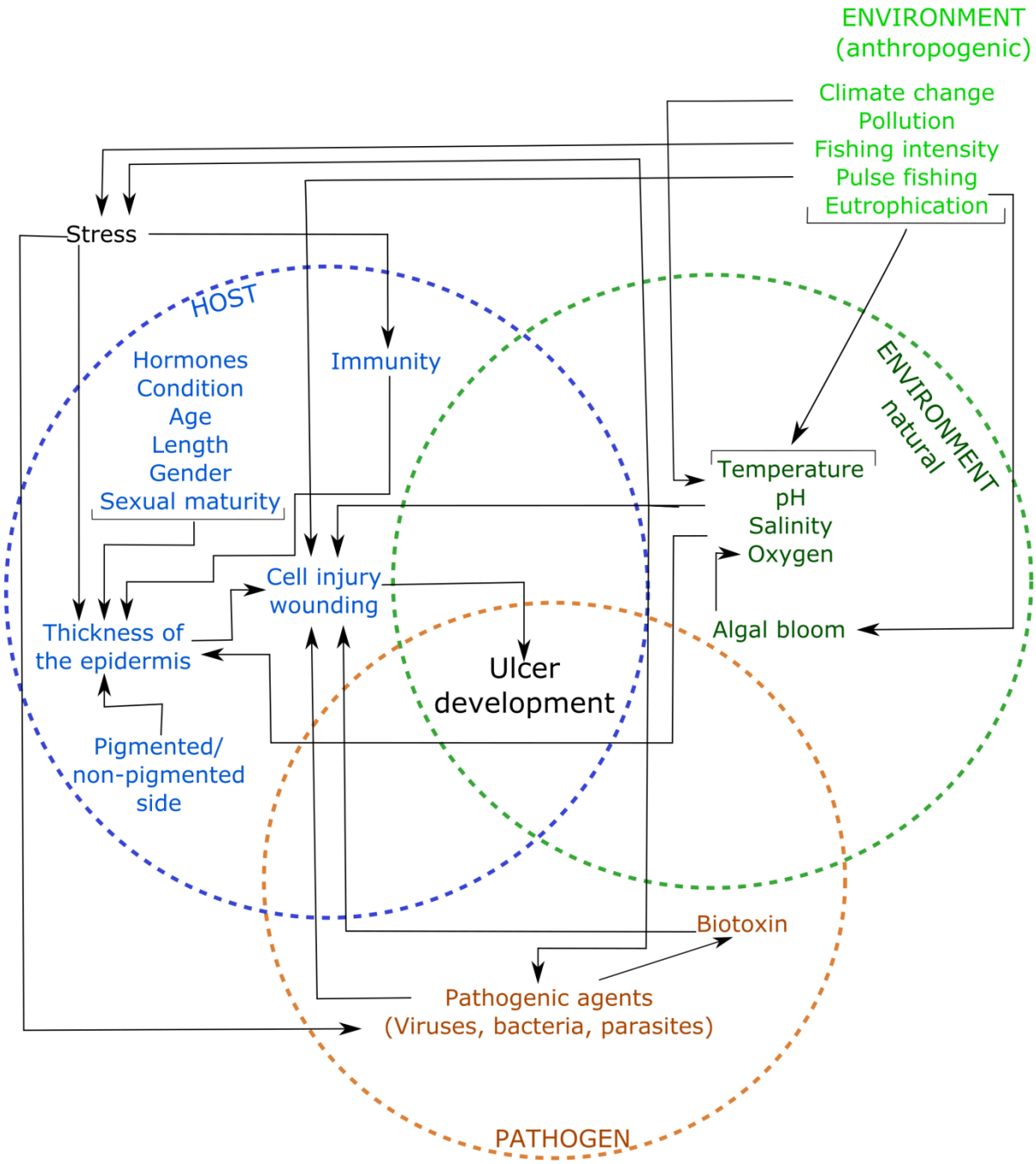

Figure 1.6: Schematic overview of all correlations between fish-related, environmental and pathogenic factors, possibly involved in skin ulceration development. This schematic gives an indication of the complexity of this system.

1.7 Biology and commercial value of common dab

As Mawdesley-Thomas stated (1972) "*Disease is the end result of an interaction between a noxious stimulus and a biological system and to understand disease is to understand all aspects of the biology of the species*." Therefore, a short overview of the biology of common dab is provided including morphology, geographical distribution, information regarding the life cycle and its commercial value.

1.7.1 Characteristics and habitat preferences

Common dab (Family Pleuronectidae) are benthic flatfish with a brownish color on the pigmented side. They live on a sandy seabed at a depth between 20 and 150 m (Daan *et al.*, 1990; Fishbase, 2019). The life expectancy of adults in the North Sea is 11 years (Henderson, 1998), and the maximal reported age was 12 years (Rijnsdorp *et al.*, 1992). The adults are generally between 11 and 25 cm long and can reach a maximum length of 40 cm (Fishbase, 2019). Dab has an opportunistic feeding strategy based on a wide prey spectrum (Hinz *et al.*, 2005). Adults mainly feed on Crustaceans (Gamaridae and *Crangon* spp.) and Polychaetes (*Nereis* spp*. and Lanice* spp.*)* as well as on smaller fish (*Gobius* spp. and *Clupea* spp.) and mollusks (mainly siphons). Juvenile dab also feeds on fish eggs (Braber & De Groot, 1973). Common dab has a key role in the marine ecosystem food web as both predator and prey (Seafish, 2010).

1.7.2 Geographical distribution

Common dab has a widespread geographic distribution and is found in the northeastern Atlantic, from the Bay of Biscay to Norway and Iceland including the Baltic Sea, White Sea and Barents Sea (Figure 1.7) (WGNSSK, 2015; FAO, 2019a).

Seasonal migrations between spawning grounds in the south and feeding grounds in the north were reported (Rijnsdorp *et al.*, 1992) although the migration pattern might be more complex and linked to different life stages (Gibson, 1997; Gibson *et al.*, 2015). The cues for this seasonal migration are presumably related to environmental factors such as currents and turbulence, light, temperature and salinity. However, endogenous rhythms can also be important (Gibson, 1997).

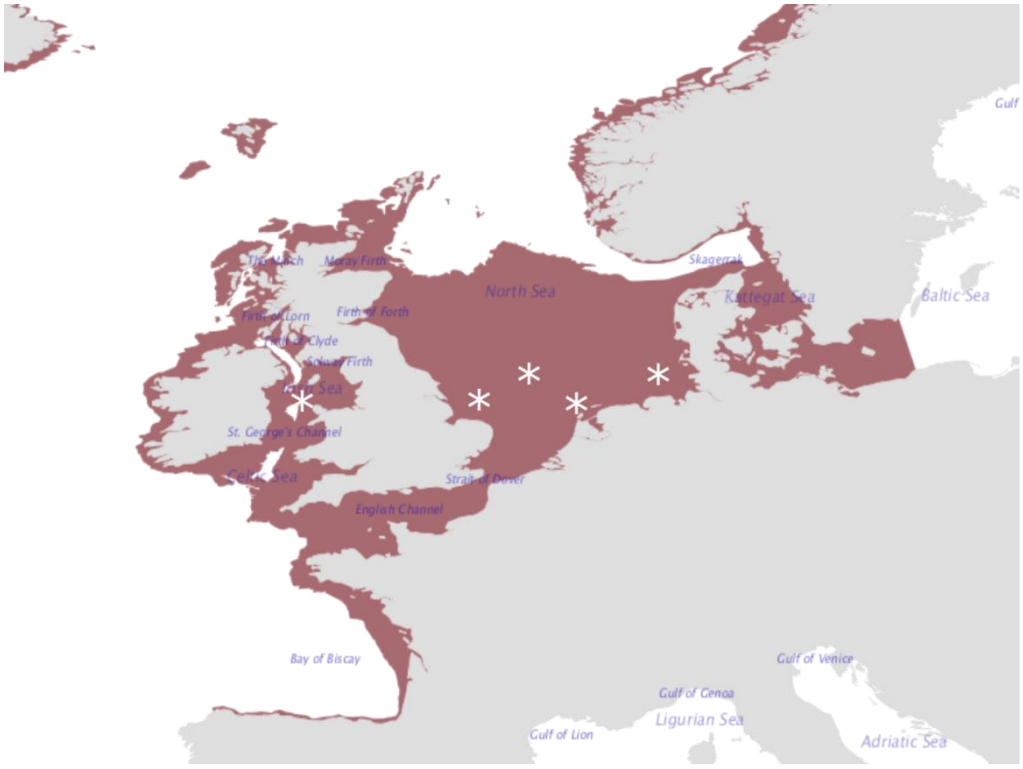

Figure 1.7: Geographical distribution of common dab (Limanda limanda) in the northeast Atlantic region. Places with an asterisk indicate the main known spawning regions (FAO, 2019a).

1.7.3 Common dab population

Common dab is one of the most abundant flatfish species in the North Sea (Daan *et al.*, 1990; WGNSSK, 2015). The wide distribution and identification of several spawning grounds indicate the presence of more than one population. It is presumed that three populations exist, one in the western British waters; one in the North Sea and Skagerrak-Kattegat; and one in the Baltic Sea (WGNSSK, 2015; Fishsource, 2019). However, it was previously also suggested that a group of dab more likely is an aggregation of fish originating from a large area or different populations (Rijnsdorp *et al.* 1992). These contradictions highlight the gap of knowledge on dab biology and distribution.

Despite the extensive exploitation, the abundancy of dab remained rather stable over the past 20 years, with some recent decreases in 2011 and 2013 but again an increase in 2014 (Hinz *et al.*, 2005; WGNEW, 2013; WGNSSK, 2015).

1.7.4 Life-history stages and linked distribution

Male common dab reaches sexual maturity approximately at 1 year (~ 11 cm), female fish between 2 and 3 years (~ 14 cm) (Rijnsdorp *et al.*, 1992). Common dab are high-fecundity serial spawners (Bolle *et al.*, 1994) and duration of spawning seasons is estimated to be five weeks for female dab. The males leave the spawning grounds later after a total spawning period of approximately 11 weeks (Henderson, 1998). Dab spawn between January and September with a peak between February and April in the Southern North Sea (Bolle *et al.*, 1994). The main known spawning regions are the German Bight, north of the Frisian Islands, the southern edge of Dogger Bank and in the northeast of Flamborough Head (Bolle *et al.*, 1994) (Figure 1.7). In the Irish Sea, also some spawning grounds exist (Beggs & Nash, 2007).

The pelagic eggs, floating in the water column, are between 0.66 and 1.20 mm in size. Dispersion of the eggs is linked to hydrodynamic, tidal and diurnal passive processes (Russell, 1976; Henderson, 1998; Beggs & Nash, 2007). This dispersion stimulates the mixing of genetic populations and subsequent gene flow (Bailey, 1997; Henderson, 1998).

Depending on the water temperature, eggs hatch after minimally 4.1 days (at 14 °C) and maximal 33 days (at 2 °C) (Beggs & Nash, 2007). In contrast to the adults, the hatched larvae resemble a typical bilaterally symmetrical fish swimming upright. During this phase, the larvae remain pelagic and perform daily vertical migrations (Gibson, 1997). The larval stage is a vulnerable stage with main mortalities linked to predation (Beggs & Nash, 2007) or pathogens (Henderson, 1998).

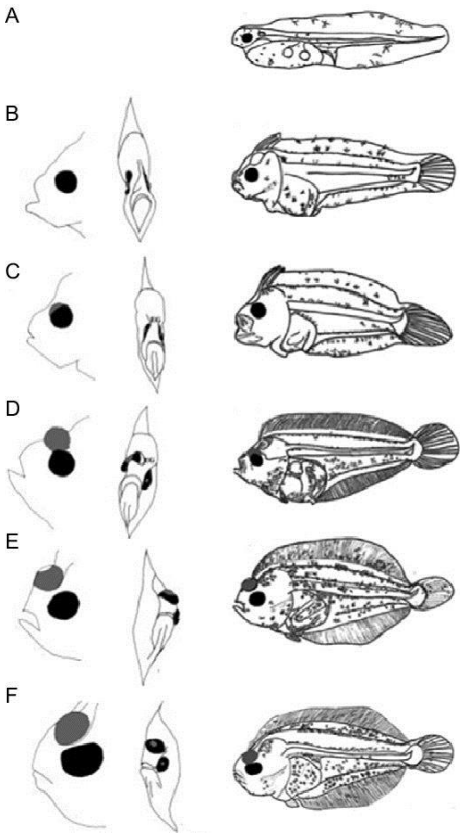

Figure 1.8: Metamorphosis of summer flounder (Paralichthys dentatus) with the migrating eye shaded in grey. The metamorphosis of summer flounder is comparable with the metamorphosis in dab, the only difference is that in dab, the left eye will migrate to the right side. The left and middle column show a schematic view of the eye migration, the right column shows associated whole-body changes (A) Hatched larvae with yolk sac. (B) Pre-metamorphosis larvae with the eyes still on both sides, symmetric larvae. (C) Early metamorphosis characterized by the start of eye movement. (D) Mid-metamorphosis. (E) Late-metamorphosis, the eye has migrated over the dorsal midline. (F) Young juvenile. Adapted from McMenamin & Parichy (2013); adapted from Martinez & Bolker (2003).

After the larval stage, a transitional stage in post-embryonic development is started, the metamorphosis. The initiation of this process is linked to body length or nutritional factors (Lund, 2007). The metamorphosis is characterized by the movement of one eye to the ocular side accompanied by drastic morphological and physiological changes such as craniofacial remodeling (Figure 1.8) and asymmetric pigment development. This ultimately results in a 90° rotation of the body, lateralized swimming posture and associated drastic deviation from the symmetric body plan.

This body rotation is associated with a behavioral and ecological adaptation, the change from a pelagic to a benthic lifestyle, also called settlement (Geffen *et al.*, 2007). The settlement in common dab is usually observed in fish with a body length of 13 to 20 mm, in the late spring or early summer (Bolle *et al.*, 1994; Amara *et al.*, 2001; Beggs & Nash, 2007). Settlement of juvenile flatfish mostly occurs in nursery grounds although for dab, no clearly delineated nursery grounds have been described, in contrast with other species such as plaice (Bolle *et al.*, 1994). Settlement of dab is

observed to occur mostly in shallow coastal (< 5 m) bays with peak densities up to 850 individuals per 1000 m² (Bolle *et al.*, 1994; Beggs & Nash, 2007). However, offshore settlement has also been described for common dab (Bolle *et al.*, 1994). By using both coastal and offshore nursery grounds, common dab maximizes the potential area for settlement (Beggs & Nash, 2007). Juvenile dab in offshore nursery ground might perform post-settlement migrations and move to coastal zones in autumn (Bolle *et al.*, 1994). In February, young dab migrate from coastal shallow areas; this is probably linked to decreasing temperatures in these zones (Bolle *et al.*, 1994). In the nursery areas, the young dab feed on siphons and tentacles of bivalves and Polychaeta such as Spionidae and Magelonidae (Amara *et al.*, 2001). The fish stay in the coastal zones (10 – 15 m depth) until two years, after which they join the adult populations.

1.7.5 Commercial value of common dab

Despite its abundancy, common dab has lower commercial value compared to sole or plaice, with an average price of € 0.66 per kg recorded between January and July of 2019 (Bellefroid *et al.*, 2019). The annual landings of common dab were approximately 6000 tonnes, mostly caught in ICES region IV, the region including the Belgian part of the North Sea. Dab is merely a bycatch species in fisheries directed at sole, plaice or shrimp and not a target species for the fishing industry. It is often discarded due to the low market value (WGNSSK, 2015). Based on the ICES InterCatch data portal, in 2013, in total 68 395 tonnes of common dab were caught worldwide of which 91 % was discarded, with majority of fish below 25 cm (WGNSSK, 2015; Seafish, 2010). Discarded dab suffers from high mortality with only a 24 % survival rate (Depestele *et al.*, 2014).

1.7 Concluding remarks

Flatfish are fascinating and important species inhabiting changing oceans. These environmental and anthropogenic changes can cause various biological effects such as increases in skin ulcerations, although exact etiology and involved risk factors are still unknown. In conclusion, the research field would benefit from increased research effort regarding the exact causality of skin ulcerations and pinpointing various risk factors that can influence the susceptibility of the fish for development of these lesions. Furthermore, the research results could offer guidance for sustainable management of the fragile marine ecosystems.

Table 1.2: Reported prevalence of skin ulcerations in common dab in various regions

Region	Year	Season	N	% Ulcerations	Reference
North Sea					
Total North Sea and Kattegat	1977-1978	Winter, spring, fall	18946	2.3 - 9.6 %	*Möller, 1979 (in Dethlefsen et al., 2000)*
Skagerrak	1983-1993	Spring	7763	0.2 - 4.4 %	*Mellergaard & Nielsen, 1997*
Southern Kattegat	1984-1993	Spring	11298	low, < 0.6 %	*Mellergaard & Nielsen, 1995*
Kattegat	1980	Summer	3187	0.6 - 2.9 %	*Möller, 1981 (in Dethlefsen et al., 2000)*
German Bight	1981-1989	Winter and Spring	41100	0.0 - 3.0 %	*Dethlefsen, 1990 (in Dethlefsen et al., 2000)*
German Bight	1983-1992	Spring	13135	0.5 - 1.9 %	*Mellergaard & Nielsen, 1997*
German Bight	1991	Spring	7565	1.7 %	*Vethaak et al., 1992*
German Bight	2008	Summer	847	0.8 %	*Lang et al., 2017*
Dogger bank	1981-1989	Winter and spring		1 - 25 %	*Dethlefsen, 1990 (in Dethlefsen et al., 2000)*
Dogger bank	1992-1997	Spring		19 %	*Dethlefsen et al., 2000*
Dogger bank (broad area)	1991-2005	Winter to spring	35961	0.5 - 3.3 %	*Vethaak et al., 2009*
Dogger bank	2008	Summer	537	± 3.9 %	*Lang et al., 2017*
Fisher bank (coastal)	1983-1992	Spring	9384	0.4 - 2.2 %	*Mellergaard & Nielsen, 1997*
Fisher bank (offshore)	1983-1992	Spring	23002	0.5 - 1.6 %	*Mellergaard & Nielsen, 1997*
Dutch coastal waters	1986-1988	Spring and Autumn	5942	0.8 %	*Vethaak & van der Meer, 1991 (in Dethlefsen et al., 2000)*
Ekofisk	2008	Summer	626	± 1 %	*Lang et al., 2017*
Southern North Sea	1981-1985	Spring autumn	16316 5439	0.3 - 1.3 % 0.8 - 3.2 %	*van Banning, 1987 (in Dethlefsen et al., 2000)*
Various regions in NS	1992-1997	Spring		< 5 %	*Dethlefsen et al., 2000*
Thames estuary	1980	Spring	363	1.4 %	*Bucke et al. 1983a*

English channel					
Rye Bay	1980	Spring	983	1.6 %	*Bucke et al. 1983a*
Various locations	1992-1997	Spring		< 5 %	*Dethlefsen et al., 2000*
Scottish east coast					
Off Firth of Forth	1987	Spring	3237	0.0 - 4.1 %	*McVicar et al., 1988 (in Dethlefsen et al., 2000)*
Firth of Forth	2008	Summer	616	± 1 %	*Lang et al., 2017*
Irish sea					
Liverpool bay	1982	Spring	3769	1.4 %	*Bucke et al., 1983b (in Dethlefsen et al., 2000)*
liverpool bay, morecambe bay	1972	Spring	936	1.0 - 1.5 %	*Shelton & Wilson, 1973 (in Dethlefsen et al., 2000)*
Various locations	1992-1997	Spring		max. 7 %	*Dethlefsen et al., 2000*
firth of solvay	1971	Spring and fall	2601	1.7 - 4.7 %	*Perkins et al., 1972 (in Dethlefsen et al., 2000)*
Iceland					
Icelandic areas	1992-1997	Spring		up to 6 %	*Dethlefsen et al., 2000*
South coast	2008	Summer	237	7.2 %	*Lang et al., 2017*
Reykjavik Bay	2008	Summer	455	± 1.9 %	*Lang et al., 2017*
Seine Bay	2008 + 2009			0.3 - 2.7 %	*Burgeot 2017*
Baltic sea					
Mecklenburg Bight	2008	Fall	1126	± 0.8 %	*Lang et al., 2017*

Chapter 2

Aims of the study

Skin ulcerations are frequently recorded lesions in wild flatfish. Based on the previously demonstrated association between development of diseases and environmental stressors, skin ulcerations in the marine environment may be used as proxy for the health status of the ecosystem. Despite the importance of these lesions and the great effort in research investigating skin ulcerations, their exact cause remains unraveled.

An explanation for this existing gap of knowledge can be found in the complexity of the fish, the ecosystem and their interactions. Moreover, since the occurrence of a disease mostly can be linked to a complex interplay between pathogens, fish and the environment, skin ulcerations are assumed to have a multifactorial cause indicating an imbalance of these factors. This complexity and the presumed multifactorial disease etiology urge for an integrated, comprehensive and multidisciplinary approach including pathogenic agents, fish-related characteristics and anthropogenic and natural environmental factors.

Considering the above, the **overall aim** of this dissertation is to increase the scientific understanding of the etiology of skin ulcerations in common dab (*Limanda limanda*) from the Belgian part of the North Sea based on a multidisciplinary study, combining regular surveys and experimental studies.

At the start of this PhD study, a survey was performed in the Belgian part of the North Sea aiming to be a first exploration of skin ulceration in common dab. Wild flatfish displaying skin ulcerations were inspected and thoroughly sampled (**Chapter 3**).

Subsequently, **specific objectives** were set in order to study **fish-pathogen interactions**. The specific objectives regarding this part of the PhD study were defined as:

> to verify the role of *Vibrio tapetis* and *Aeromonas salmonicida* in the development of skin ulcerations in wild-caught common dab, by performing an *in vivo* experimental challenge. Both bacterial species were isolated from active skin ulcerations during the explorative survey (**Chapter 4 and 6**);
>
> to investigate the plausible fish-related variability between isolates of *V. tapetis* retrieved from various bivalves and fish species. Three methodologies were compared and evaluated for their ability to discriminate the potential virulence against the Manila clams (**Chapter 5**).

As mentioned before, it is assumed that various factors may interact and have a synergistic or additive effect on the development of skin ulcerations (See 1.5.3.4). Aims regarding the **integrated research approach** on skin ulcerations were defined as:

> to gather more information regarding the pathogens, fish-related and environmental factors involved in skin ulceration development via a bi-monthly survey in the Belgian part of the North Sea, performed between 2016 and 2019. During these surveys we aimed to further substantiate the role of both *V. tapetis* and *A. salmonicida* in skin ulcerations in common dab and gather information on other bacterial species present in the lesions. Furthermore, the impact of predisposing fish-related factors such as the body condition, sex and length in the development of skin ulcerations in common dab was assessed and correlations between environmental factors and changing prevalence of skin ulcerations in common dab were studied as a mean to predict the predisposing role of the environment in the development of skin ulcerations (**Chapter 7**).

to develop an innovative two-chamber skin explant model, specifically designed for marine fish, with the possibility to integrate pathogenic agents, fish-related factors and environmental factors in one *in vitro* experiment. This model aimed to be a comprehensive tool in further research on the etiology of skin ulcerations and other skin lesions **(Chapter 8)**.

Part I

Explorative study

Chapter 3

Explorative study on skin ulcerations

Based on: Vercauteren, M., De Swaef, E., Declercq, A. M., 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.

3.1 Abstract

Skin ulcerations rank amongst the most prevalent lesions affecting wild common dab (*Limanda limanda*) with an increase in prevalence of up to 3.5 % in the Belgian part of the North Sea. A complex etiology of these ulcerations is suspected and many questions remain on the exact factors contributing to these lesions. To construct the etiological spectrum of skin ulcerations in flatfish, a one-day monitoring campaign was undertaken in the North Sea. Fifteen fish presented with one or more ulcerations on the pigmented and/or non-pigmented side. Pathological features revealed various stages of ulcerations with loss of epidermal and dermal tissue, inflammatory infiltrates and degeneration of the myofibers bordering the ulceration, albeit in varying degrees. Upon bacteriological examination, pure cultures of *Vibrio tapetis* were retrieved in high numbers from five fish and of *Aeromonas salmonicida* in one fish. The *V. tapetis* isolates showed cross-reactivity with the sera against the representative strain of serotype O2 originating form a carpet-shell clam (*Ruditapes descussatus*). Moreover, the *A. salmonicida* isolates displayed a previously undescribed *vapA* gene sequence (A-layer type) with possible specificity towards common dab. Further research is necessary to pinpoint the exact role of these agents in the development of skin ulcerations in common dab.

3.2 Introduction

Skin ulcerations in flatfish, defined as lesions whereby the epidermis and dermis are affected, are reported in different areas worldwide, with a prevalence generally ranging from 0 to 9 % (Vethaak, 1992; Wiklund & Bylund, 1993; Wiklund, 1994; Mellergaard & Nielsen, 1997; Law, 2001). As part of the national monitoring campaigns to assess the health status of wild fish, half-yearly sea-trips in the Belgian part of the North Sea were undertaken. These revealed an increase in the number of common dab (*Limanda limanda*) exhibiting skin ulcerations from 2011 onwards, with up to 3.5 % of the sampled animals affected (Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), pers. comm.). In addition to common dab, other species such as plaice (*Pleuronectes platessa*), turbot (*Scophthalmus maximus*), whiting (*Merlangius merlangus*) and European flounder (*Platichthys flesus*) may develop skin ulcerations (Wiklund, 1994; Vethaak, 2013). With respect to the impact of these ulcerations, a compromised skin barrier may be associated with reduced survival, which may increase the mortality of the fish and thus reduce the productivity of the stock. In addition, this added mortality is unaccounted for in the mortality data used in stock assessment.

Skin ulcerations in wild fish often have a complex etiology and hitherto, the knowledge on the possible mechanisms involved is meagre (Lang *et al.*, 1999; Stentiford *et al.*, 2009). Three categories of factors are presumed to play a role in the development of skin ulcerations. A first category consists of environmental factors such as temperature (Wiklund & Bylund, 1993; Mellergaard & Nielsen, 1997), salinity fluctuation (Mellergaard & Nielsen, 1997; Vethaak, 2013) and oxygen depletion (Mellergaard & Nielsen, 1997; Law, 2001). Secondly, fish-related factors such as sex, length and previous trauma may also have an effect (Wiklund & Bylund, 1993; Mellergaard & Nielsen, 1997; Law, 2001; Vethaak *et al.*, 2009). The last category encompasses micro-organisms, which are known to potentially cause skin ulcerations or aggravate existing lesions, including fungal species (Vogelbein *et al.*, 2001), viruses (Jensen & Larsen, 1982), parasites (Law, 2001) and bacteria (López *et al.*, 2011), with the latter forming the majority (Wiklund *et al.*, 1999). Various bacterial species such as *Mycobacterium ulcerans* (Eddyani *et al.*, 2004), *Vibrio* spp. (Colwell & Grimes, 1984), and *Aeromonas* spp. (Wiklund, 1990; Magnadottir *et al.*, 2002) have been associated with ulcerative diseases in wild fish.

Nevertheless, the key question on the exact role of these bacterial agents and other environmental or fish-related factors in the development of skin ulcerations in wild fish remains largely unanswered (Law, 2001). This lack of knowledge is rooted in the suspected multifactorial etiology of these lesions. For that purpose, in the present study, wild flatfish procured from the Belgian part of the North Sea displaying skin ulcerations were inspected and thoroughly sampled. In order to further enable elucidation of the cause(s) of such lesions and to minimize the confounding effects of post-mortem degeneration and saprophytic colonization (Khuntia, 2009), a full necropsy and sampling were performed on freshly sacrificed fish.

3.3 Materials and methods

3.3.1 Animals and retrieval sites

Common dab were caught on a one-day campaign on board of the research vessel 'Simon Stevin' using a 3 meter beam trawl at three locations situated in the western sector of the Belgian part of the North Sea, approximately 5 km from one another (Figure 3.1).

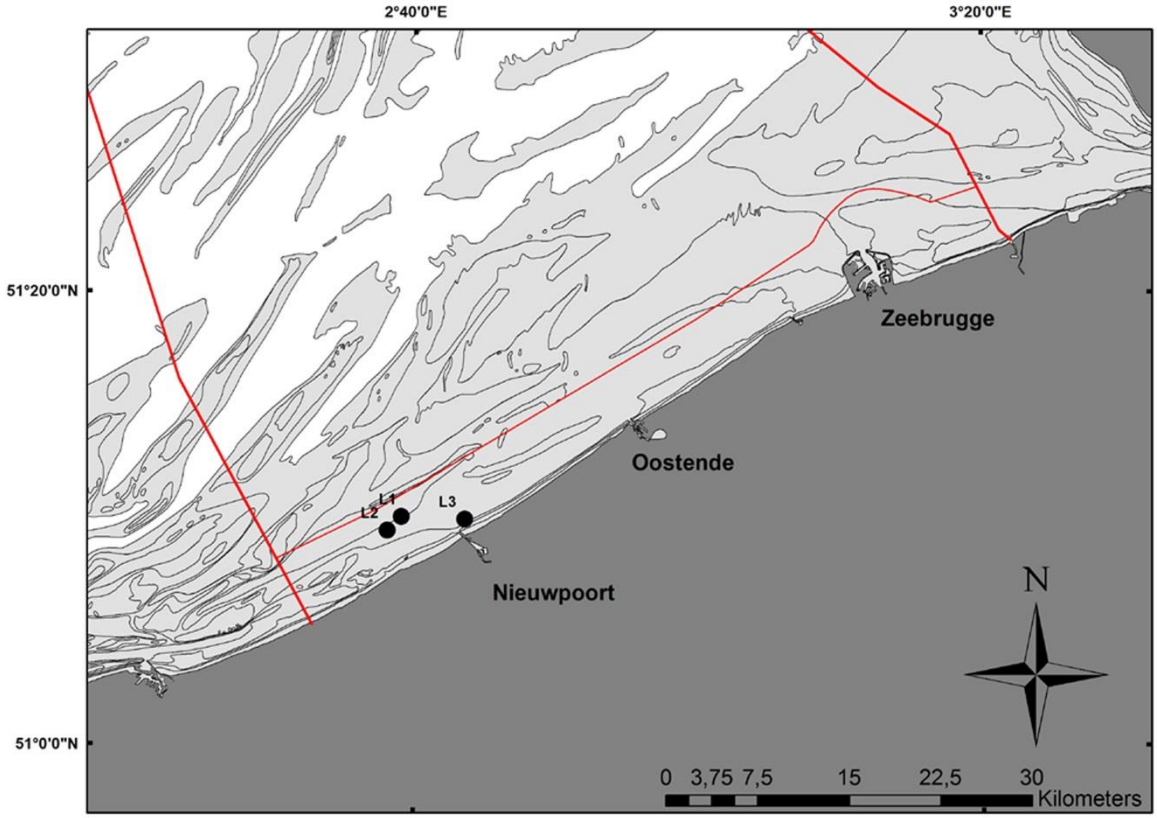

Figure 3.1: Map of the retrieval sites (L1 (N51°10'10.56; E2°39'13.02), L2 (N51°9'49.14; E2°38'13.02) and L3 (N51°10'0.00; E2°43'60.00)) in the Belgian part of the North Sea with the red lines indicating the border of the Belgian part of the North Sea and the reference boundary with a distance of 3 sea miles off shore.

Short fishing hauls with duration of approximately 30 minutes were carried out. Dab displaying skin ulcerations were euthanized on board of the research vessel with an overdose of benzocaine (ethyl 4-aminobenzoate, 1 g per 10 ml ethanol; Merck, Germany), immediately inspected and sampled as described below. The temperature, salinity, depth and oxygen saturation of the water at the retrieval sites were registered by means of a Conductivity-Temperature-Depth (CTD)-probe (Seabird 19plus V2, Sea Bird Electronics, USA).

The sampling was carried out in accordance with the approved guidelines and legislation in force with regard to animal welfare.

3.3.2 Examination and sampling of ulcerations

The ulcerations were photographed (Canon PowerShot G16, Canon), their occurrence (pigmented/non-pigmented side) and diameter were recorded. Based on their macroscopic appearance, ulcerations were classified as 'ulceration', 'partially healed ulceration' or 'completely healed ulceration' following the guidelines compiled by the International Council for Exploration of the Sea (ICES) (Bucke *et al.*, 1996).

Samples for bacteriological analysis were taken from the edges of one ulceration per fish and inoculated onto Marine Agar (Scharlau Microbiology, Spain) and modified Shieh agar plates supplemented with 1.5 % NaCl (Shieh, 1980; Song *et al.*, 1988) which were aerobically incubated at 17 ± 1 °C for 7 days. For histological examination, samples were fixated for 24 h in a phosphate-buffered 4 % formaldehyde solution, dehydrated in an alcohol-xylene series and embedded in paraffin wax. All tissues were sectioned (5 µm, Microm HM360, Microm Inc. USA) and stained with hematoxylin and eosin (H&E). In

addition, immunohistochemistry was performed to detect the presence of *V. tapetis* and/or *A. salmonicida*. For that purpose, 5 µm sections were made on APES-coated slides (APES, Sigma-Aldrich NV/SA, Bornem, Belgium) and dried during one hour (60 °C) and overnight (37 °C). After dewaxing with xylene and rehydration in series with ethanol and distilled water, the sections were incubated in the pressure cooker with a citrate buffer (pH 6) for epitope exposure. Subsequently, primary antibodies (Rabbit Primary Antibodies, DAKO) were added using the DAKO Envision+ System/HRP diaminobenzidine (DAB+) kit (DAKO, Agilent Technologies Inc., USA), according to the instructions of the manufacturer. Polyclonal antibodies against *V. tapetis* (Biochemistry and Molecular Biology Department, CIBUS, University of Santiago de Compostela, Spain) or monoclonal antibodies against *A. salmonicida* (FM-020-AY-5, Austral biologicals, California, USA) were added with a dilution of 1:500 and 1:100, respectively. Thereafter, the sections were counterstained with Mayer's hematoxylin and mounted. Subsequently, a full necropsy was performed.

3.3.3 Identification and typing of bacterial isolates

The genomic DNA of all retrieved isolates was extracted according to Declercq *et al*. (2013) and the 16S rRNA gene was amplified (Smet *et al*., 2012). Finally, the genetic sequences were determined and compared to sequences in the NCBI /GenBank and Greengenes databases using the similarity search program BLAST.

Eight isolates identified as *V. tapetis* using 16S rRNA sequencing, were submitted for serotyping (Table 3.1). For that purpose, bacterial colonies were typed by a rapid slide agglutination test using antisera raised against three *V. tapetis* strains, isolated from, respectively, Manila clam (*Ruditapes philippinarum*; CECT 4600^{T} genotype / serotype O1)*, c*arpet-shell clam (*R. descussatus;* GR0202RD genotype /serotype O2) and Atlantic halibut *(Hippoglossus hippoglossus;* HH6087 genotype/serotype O3) (Rodriquez *et al.*, 2006; Balboa *et al.*, 2011)*.*

All six isolates identified as *A. salmonicida* using 16S sequencing were subjected to sequence analysis of the virulence array protein (*vapA*) gene (± 1500bp), which encodes for the A-layer protein (Chu *et al.*, 1991). In accordance with a previously published typing scheme (Gulla *et al.*, 2016), a hypervariable region of the *vapA* gene was amplified and sequenced using primers F2 and R3.

3.4 Results

The depth, temperature, salinity, and oxygen saturation of the three sampling locations varied between 8.16 and 13.13 m, 11.71 and 12.05 °C, 34.19 and 34.24 practical salinity unit (PSU) and 76.49 % and 79.79 % oxygen, respectively.

In total, 370 dab were caught, out of which 15 fish (ten males and five females) displayed one or more skin ulcerations. The lesions were present both on the pigmented and non-pigmented side, with no specific predilection site. A general overview of the dab with skin ulcerations, characteristics of the fish, ulcerations, retrieval site and results of the bacteriological analysis is listed in Table 3.1.

Thirteen skin lesions consisted of sharply delineated hemorrhagic skin ulcerations, ranging between 0.31 and 1.04 cm in diameter, with adjacent cutaneous petechial hemorrhages. These hence were classified as "ulceration" (Figure 3.2A). In six lesions, the border of the ulceration was not sharply delineated and displayed a variable-sized white rim devoid of scales (classified as "partially healed ulceration") (Figure 3.2B). The epidermis of one lesion was covered with a white, scale-less scar (classified as "completely

healed ulceration") (Figure 3.2C). The partially and completely healed ulcerations had a diameter of 0.64 ± 0.44 cm and hemorrhages in the adjacent skin were absent.

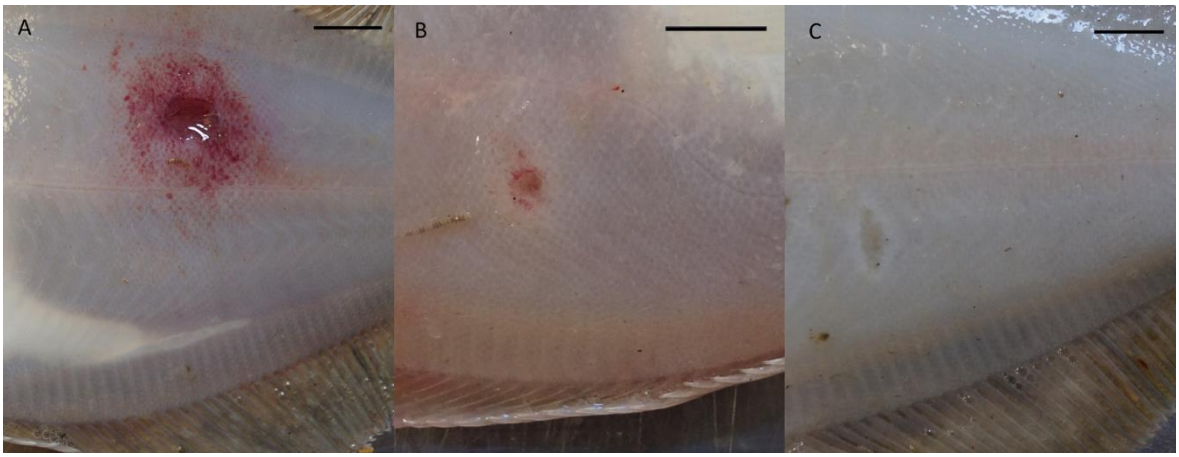

Figure 3.2: Classification of skin ulcerations located on the non-pigmented side of common dab (Limanda limanda) based on their macroscopic appearance. (A) Ulceration. Note the petechial hemorrhages adjacent to the lesion. Bar = 1 cm. (B) Partially healed ulceration. Note the white rim indicative of healing. Bar = 1 cm. (C) Completely healed ulceration recognizable as a white scale-less scar. Bar = 1 cm.

No abnormalities of the internal organs were noted upon post-mortem examination.
Upon histological examination, the skin ulcerations were characterized by a complete loss of epidermis and scales with exposure of dermal collagen. The dermis was infiltrated by mild to moderate amounts of inflammatory cells and scattered extravasated red blood cells were present (hemorrhages) (Figure 3.3A). Underlying muscle tissue was often moderately degenerated and/or infiltrated by inflammatory cells and the adjacent epidermis was frequently hyperplastic. Completely healed ulcerations had an intact epidermis with disrupted stratum compactum and stratum spongiosum. Scattered inflammatory cells were found in adjacent dermis and superficial muscular tissue (Figure 3.3B). In partially healed ulcerations, a mix of the above described changes was observed. Immunohistochemical staining revealed the presence of *V. tapetis* and *A. salmonicida* in epidermal and dermal tissues of two and three ulcerations, respectively.
The results of the bacteriological findings are presented in Table 3.1. Following 48 h incubation of the Marine Agar and supplemented Shieh medium inoculated with the samples of the ulcerations of four fish (fish number 1, 5, 7, 14), an abundant culture of white-yellowish colonies between 2 and 3 mm with a smooth edge appeared. Pure cultures emerged on both agar types of five fish (fish number 2, 4, 10, 11, 13). Sequencing of the 16S-rRNA gene led to the identification of *Vibrio tapetis* with 99 % sequence homology.

Additionally, after one week of incubation, small (≤ 1 mm), 'friable', white colonies appeared on the supplemented Shieh medium plates inoculated with the samples of six fish (fish number 1, 3, 6, 7, 9 and 12). These isolates were identified as *A. salmonicida* using 16S rRNA sequencing. In the ulceration of one fish (fish number 12), a pure culture of *A. salmonicida* was detected whereas in the other five animals (fish number 1, 3, 6, 7 and 9) *A. salmonicida* was recorded together with *V. tapetis* and/or another bacterial species (Table 3.1).

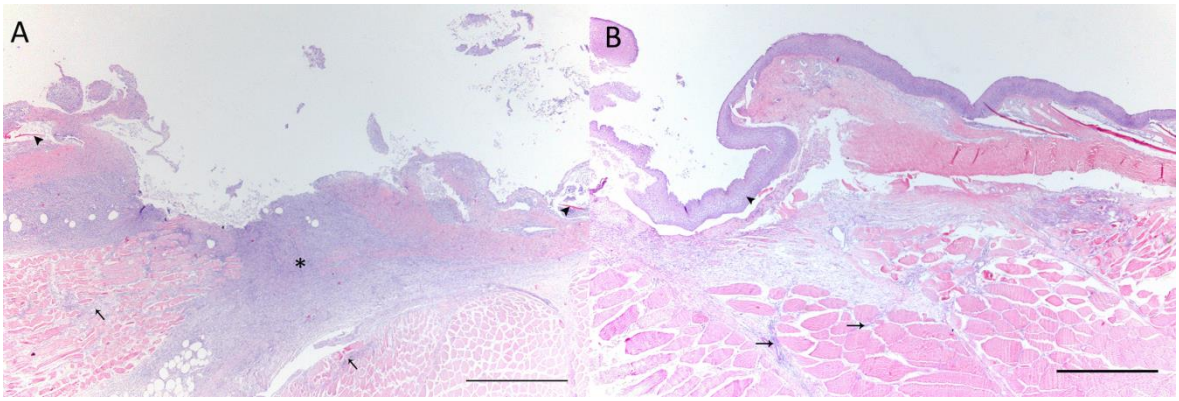

Figure 3.3: Histological appearance of the various ulceration types encountered in the sampled common dab (Limanda limanda). (A) Histologic section of a skin ulceration with transition to intact skin, as exhibited by the presence of scales (arrowhead). The epidermis and dermis are lost with degeneration of underlying muscle (arrow). A severe inflammation (*), even extending between muscle fibers, is present. H&E, bar = 1mm. (B) Histologic section of a completely healed skin ulceration. Note the re-epithelialization (arrowhead) with disrupted stratum compactum and stratum spongiosum as well as the absence of scales. A moderate amount of inflammation is present in dermis and between muscle fibers (arrow). H&E, bar = 1mm.

In addition to *V. tapetis* and *A. salmonicida*, other bacterial species such as *V. splendidus*, *Aliivibrio wodanis, Pseudoalteromonas* species (*P. espejinana*, *P. haloplanktis, P. atlantica*), and *Shewanella frigidimarina* were identified using 16S sequencing. However, these were always part of mixed cultures and never abundantly present (Table 3.1).

All retrieved *V. tapetis* isolates showed cross-reactivity with the antiserum against the carpet-shell clam strain (genotype /serotype O2), except for one isolate retrieved from the skin ulceration from fish number 2 which displayed a strong reaction with antisera against both serotype O2 and O3, originating from the Atlantic halibut strain.

The partial *vapA*-sequences generated from six isolates identified as *A. salmonicida* showed ≤ 90 % homology with all 14 previously characterized and described A-layer types (Gulla *et al.,* 2016). Moreover, amongst the six isolates more than 99 % sequence homology was found, with point mutations appearing at four nucleotide positions (85, 88, 89, and 287).

Table 3.1: Results of gross and bacteriological examination of common dab (Limanda limanda) with skin ulcerations.

				Ulceration				Bacteriological analysis		
Fish number	Length (cm)	Sex	No. of ulceration	Location	Type	Diameter (cm)	Retrieval site	*Aeromonas salmonicida*	*Vibrio tapetis*	Other bacterial species
1	17.8	M	1	NP	U	0.7	L1	+	+"	+
2	14.8	M	1	NP	U	0.5	L1	-	+"	-
3	12.0	M	1	P	U	0.6	L1	+	-	+
4	16.0	F	1	NP	U	0.8	L1	-	+"	-
5	16.6	F	1	NP	PH	0.5	L1	-	+"	+
6	20.0	M	3	P	U	1.1	L1	+	-	+
				NP	PH	0.8		NS	NS	NS
				NP	PH	0.9		NS	NS	NS
7	15.7	M	2	NP	U	0.3	L1	+	+	-
				NP	PH	0.2		NS	NS	NS
8	15.8	M	1	NP	U	1.0	L1	-	-	+
9	15.4	M	1	NP	PH	0.3	L2	+	-	+
10	15.0	M	1	P	U	0.6	L2	-	+"	-
11	16.9	M	1	NP	U	0.8	L2	-	+"	-
12	25.2	F	1	P	U	1.0	L2	+	-	-
13	17.7	M	2	NP	U	0.6	L2	-	+"	-
				NP	C	1.5		NS	NS	NS
14	17.8	F	2	NP	U	0.7	L2	-	+"	+
				NP	PH	0.3		NS	NS	NS
15	21.3	F	1	NP	U	0.6	L3	-	-	+

Length (cm), sex (Male (M) – Female (F), ulceration characteristics (number, location (P: pigmented side; NP: non-pigmented side), type (U: ulceration; PH: partially healing ulceration; C: completely healed ulceration), diameter), retrieval site and results of the bacteriological analysis of the 15 common dab (Limanda limanda) displaying skin ulcerations. +: present; -: Not present; NS: not sampled; V. tapetis isolates marked with an quotation mark were used for serological typing.

3.5 Discussion

Skin ulcerations rank amongst the most prevalent lesions affecting wild fish and preoccupy the scientific and sea-guarding communities. These concerns are justified as skin ulcerations may result in a loss of appetite (Vilar *et al.*, 2012), change in swimming behavior (Vilar *et al.*, 2012), loss of reflexes (Davis & Ottmar, 2006), osmotic disbalance (Mellergaard & Nielsen, 1997; Vilar *et al.*, 2012), decreased condition of the fish (Mellergaard & Nielsen, 1997) and even death (Mellergaard & Nielsen, 1997).

Despite their importance, these skin lesions were previously merely scored macroscopically using a crude semi-quantitative scoring system of the skin ulcerations based on their presence, intensity and impact on the host. The scoring of skin ulcerations is part of the calculated Fish Disease Index (FDI) used as indicators for the health of the marine environment (Bucke *et al.*, 1996; Lang *et al.*, 2017). This superficial scoring was overcome in the present study, i.e. by sampling freshly sacrificed fish on board of the research vessel and perform a thorough sampling to avoid the confounding effects of post-mortem degeneration, bacterial overgrowth (Khuntia, 2009) and generating a broad database.

This methodology resulted for the first time in an abundant and therefore probably clinically relevant isolation of *V. tapetis* and *A. salmonicida* from skin ulcerations of wild-caught common dab.

Vibrio tapetis was first described by Paillard & Maes (1990a) as the causative agent of brown ring disease occurring in various clam species (Paillard, 2004a). Over the last decade, *V. tapetis* has also been isolated from cultivated or captive held marine species, including corkwing wrasse (*Symphodus melops*) (Jensen *et al.*, 2003), Atlantic halibut (Reid *et al.*, 2003), wedge sole (*Dicologlossa cuneata*) (López *et al.*, 2011) and Dover sole (*Solea solea*) (Declercq *et al.*, 2015). An infection with *V. tapetis* may result in death (Reid *et al.*, 2003; Jensen *et al.*, 2003, López *et al.*, 2011), loss of appetite (Jensen *et al.*, 2003), development of vesicular lesions (Declercq *et al.*, 2015) and/or ulceration development in captive or cultivated fish. In the rapid slide agglutination tests, all *V. tapetis* isolates from the wild-caught common dab of this study showed reactivity with antisera against the representative strain of *V. tapetis* serotype O2 (GR0202RD), originating from carpet-shell clam species. These results are comparable to what was found for the bacterial isolates retrieved from vesicular lesions in Dover sole (Declercq *et al.*, 2015) and from the wedge sole (López *et al.*, 2011) which also belonged to serotype O2. Noteworthy, the isolate coming from one ulceration showed additional reactivity with antiserum against the representative strain of serotype O3 (HH6087), originally isolated from Atlantic halibut (Reid *et al.*, 2003; Declercq *et al.*, 2015). It would be interesting to further investigate the heterogeneity of fish and clam isolates of *V. tapetis* and study the host-specificity with special attention for pathogenicity.

Various *Aeromonas salmonicida* subspecies have been isolated from skin ulcerations in fish species such as salmonids (*Salmonidae*) (Shieh & Maclean, 1975; Wiklund & Dalsgaard, 1998), goldfish (*Carassius auratus*) (Elliott & Shotts, 1980), European flounder (Wiklund, 1995), turbot (Toranzo & Barja, 1992; Pedersen *et al.,* 1994), pike (*Esox* sp.) (Wiklund, 1990), and common dab (Wiklund & Dalsgaard, 1995; Wiklund *et al.*, 1999). *A. salmonicida* virulence has been linked to the outer membrane which is a paracrystalline protein with a molecular mass of approximately 50 kDa (Kay *et al.,* 1981; Chu *et al.*, 1991). It is believed to protect the bacterial cells against the serum complement-mediated killing (Munn *et al.,* 1982) and protease degradation (Kay & Trust, 1991). Moreover, it may facilitate intracellular macrophage survival (Daly *et al.,* 1996).

The hypervariable *vapA*-region sequenced in the present study encodes a surface-exposed and presumed antigenic proportion of the A-layer protein, which may be of importance for host-pathogen interactions (Gulla *et al.*, 2016). Previously published A-layer types have displayed varying degrees of host predilection, and all *A. salmonicida* isolates recovered in this study belong to a previously unknown and undescribed A-layer type, proposed here as A-layer type 15. Conceivably, this A-layer type may represent an *A. salmonicida* subtype with specificity towards common dab, and further examination of spatiotemporally unrelated flatfish isolates (Gulla *et al.*, un-published data) supports this. Species-specific susceptibility to *A. salmonicida* might also be reflected in varying clinical signs and pathogenicity,

a hypothesis which already was rectified for other fish species (Obradovic, 1983; Pedersen *et al.*, 1994; Wiklund & Dalsgaard, 1998; Magnadottir *et al.*, 2002; Toranzo *et al.*, 2005; Carson & Handlinger, 2006; Gulla *et al.*, 2016).

In conclusion, this research signifies a step further towards unravelling the most likely multifactorial etiology of skin ulcerations in fish, with the isolation of *V. tapetis* and *A. salmonicida,* the latter with a hitherto undescribed A-layer type, possibly implying species-specificity. Both agents may have played a role in the development of the skin lesions observed. However, further studies are needed as any pathological association between the noted lesions and the above agents remains fairly speculative.

3.6 Acknowledgements

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Part II

Fish–pathogen interactions

Chapter 4

Role of *Vibrio tapetis* in skin ulceration development

Based on: Vercauteren, M., De Swaef, E., Declercq, A. M., Polet, H., Aerts, J., Ampe, B., Romalde, J. L., Haesebrouck, F., Devriese, L., Decostere, A. and Chiers, K. 2019. Scrutinizing the triad of Vibrio tapetis, the skin barrier and pigmentation as determining factors in the development of skin ulcerations in wild common dab (*Limanda limanda*). *Veterinary Research*, 50: 41.

4.1 Abstract

Recently, *Vibrio tapetis* was isolated for the first time from skin ulcerations in wild-caught common dab (*Limanda limanda*). To further examine its role in the development of these skin lesions, an *in vivo* experiment was performed. The significance of the skin barrier and in addition the difference between pigmented and non-pigmented side were investigated. Hence, the skin of common dab was treated in three different ways on both the pigmented and non-pigmented side. On a first 'treatment zone', the scales and overlying epidermal tissue were removed whereas in a second zone only the mucus was discarded. The third zone served as a non-treated zone. Thereafter, fish were challenged with *V. tapetis*. The control group was sham treated. Mortality, clinical signs, severity and size of the developing lesions were recorded. All animals were sacrificed and sampled 21 days post inoculation.

Significantly more fish of the group challenged with *V. tapetis* died compared to the control group with the highest incidence occurring 4 days post inoculation. Fish challenged with *V. tapetis* developed more severe skin ulcerations. In zones where scales and epidermal tissue were removed, the ulcerations were more severe compared to zones where only mucus was eliminated. Ulcerations occurred more frequently, were more severe and larger on the pigmented side.

Our data represents prove of *V. tapetis* as causative agent of ulcerative skin lesions although prior damage of the skin seems to be a major contributing factor. Furthermore, the pigmented side seemed predisposed to the development of skin ulcerations.

4.2 Introduction

Vibrio tapetis is a well-known pathogen causing brown ring disease in various clam species such as the manila clam (*Ruditapes philippinarum*) and carpet-shell clam (*R. decussatus*) (Paillard & Maes, 1994; Paillard, 2004a). Only a handful of studies describe the isolation of *V. tapetis* from skin lesions and internal organs of moribund cultivated or captive held aquatic vertebrates, namely corkwing wrasse (*Symphodus melops*) (Jensen *et al.*, 2003), Atlantic halibut (*Hippoglossus hippoglossus*) (Reid *et al.*, 2003), wedge sole (*Dicologlossa cuneata*) (López *et al.*, 2011), Dover sole (*Solea solea*) (Declercq *et al.*, 2015) and two native fish species reared in Chile (*Genypterus chilensis* and *Paralichthys adspersus*) (Levican *et al.*, 2017). Recently, *V. tapetis* was isolated for the first time from skin ulcerations, lesions affecting epidermal and dermal tissues, in wild-caught common dab (*Limanda limanda*) in the Belgian part of the North Sea (BNS) (Vercauteren *et al.*, 2017).

The wild common dab populations in this geographic area showed a recent prevalence of skin ulcerations up to 3.5 % (Devriese *et al.*, 2015). Various possible causal agents, ranging from infectious agents to biological toxins, were suggested as involving factors in the development of skin ulcerations (Wiklund, 1994; Noga, 2000; law, 2001). Nevertheless, the etiology of these lesions is largely unknown and many questions remain regarding their development and possible contributing factors in terms of fish health and environmental conditions. Besides being a welfare issue, skin ulcerations in wild fish may also pose limitations to the productivity of the marine ecosystem (Bouck & Smith, 1979; Noga, 2000) and diminish the economic value of the fish (Noga, 2000), urging for an understanding of the cause(s) of these lesions.

All marine organisms, including fish, live in close contact with their environment, containing potential pathogenic micro-organisms (Ellis, 2001). Hence, the skin acts as an important barrier between the fish and its environment. The outermost part of the skin consists of the epidermal layer and the covering mucus layer; two complex structures offering mechanical, chemical and immunological protection against pathogenic agents (Svendsen & Bøgwald, 1997; Raj *et al.*, 2011). Changes in one or both layers, caused by abrasion, stress or pollutants, may increase the susceptibility of the fish for adherence and subsequent invasion of microorganisms and hence for the development of skin ulcerations, as demonstrated for *Tenacibaculum maritimum* in four marine species (Chen *et al.*, 1995).

Common dab and flatfish in general, have a fiercely discussed asymmetric morphology, with the pigmented (P) side facing the water column and the non-pigmented (NP) side being in closer interaction with the sediment (Spinner *et al.*, 2016). Based on these differences, a divergent morphological composition and possibly linked varying susceptibility to the development of skin ulcerations is plausible. A handful of studies on this issue are available but inconsistent ranging from a clear to a sex-related difference regarding the development of skin ulcerations on both sides (Vethaak, 1992; Wiklund & Bylund, 1993; Mellergaard & Nielsen, 1997; Vethaak, 2013).

In view of this, the aim of the present study was to (i) pinpoint the ability of *V. tapetis* to induce skin ulcerations in common dab; (ii) assess the impact of prior skin damage on the development of skin ulcerations and (iii) determine differences between the pigmented and non-pigmented side on the development of skin ulcerations.

4.3 Materials and Methods

4.3.1 Animals and housing facilities

Sixty common dab were caught during short fishing hauls (± 10 min) using a 6 m beam trawl on board of the research vessel (RV) 'Simon Stevin' at two sampling locations (L1 (N51°10.344; E2°38.699) and L2 (N51°9.886; E2°34.797)) situated in the western segment of the BNS, within three miles offshore. On board of the RV, only fish in good condition and with a minimal length of 17.5 cm were collected and immediately placed in a survival tank (1 x 1.2 x 1 m; 640 L) with continuous water renewal. Within 4 h after capture, all fish were transferred to circular tanks (diameter 2.6 m, 4000 L; 30 fish per tank) with sand-covered bottoms (6 cm layer thickness, 0 - 2mm grain size) and filled with recirculating, filtered natural seawater at the Marine Station Ostend (Flanders Marine Institute, VLIZ, Ostend, Belgium). The water quality was monitored daily and kept in pre-set ranges (16.3 ± 0.4 °C; pH 8.1 ± 0.0; 33.8 ± 0.2 PSU; 85.3 ± 1.1 % oxygen saturation). Ammonia and nitrite levels never exceeded 0.1 and 1 mg L^{-1}, respectively. Fish were fed three times a week with chopped whiting (*Merlangius merlangus*) (10 g per fish). After an acclimatization period of 18 days, the fish were transported to the experimental units using small transportation boxes (39.4 x 59.8 x 18.6 cm; 30 L) supplied with an oxygen tablet (JBL GmbH & Co.KG, Germany).

Upon arrival at the experimental units, fish were randomly divided over five tanks (1 x 1 x 0.5 m; 450 L) with each tank containing 12 fish. The tanks were filled with recirculating and aerated seawater, half natural and half artificial (Instant ocean, Aquarium system, France) and a sand layer (6 cm layer thickness, 0 - 2mm grain size). Water parameters were monitored daily. Throughout the experiment, the following averages were recorded: 15.3 ± 1.0 °C, pH 8.4 ± 0.1; 32.5 ± 1.5 PSU and 79.5 ± 6.6 % oxygen saturation. Ammonia and nitrite levels never exceeded 0.2 and 1 mg L^{-1}, respectively. Upon water renewal, artificial seawater was added. Fish were fed every two days with chopped Pangas catfish (*Pangasius pangasius*) (10 g per fish). Feeding time lasted for one hour after which the remaining feed was removed. One week after arrival, all fish were deprived of food 24 h prior to the experimental trial. The experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine and Bio-engineering Sciences, Ghent University (EC 2015_89).

4.3.2 Bacterial isolate

The *V. tapetis* isolate was recovered in pure culture from an ulcerative skin lesion of a common dab caught in the BNS (Vercauteren *et al.*, 2017).

For the experimental challenge, the *V. tapetis* isolate was cultured on Tryptic Soy Agar (TSA, Sigma Aldrich N.V., Belgium) supplemented with 1.5 % NaCl and incubated at 16 ± 1°C for three days. Colonies were subsequently transferred to 4 mL Tryptic Soy Broth (TSB, Sigma Aldrich N.V., Belgium) supplemented with 1.5 % NaCl. After a 24 h incubation period at 16 ± 1 °C, the broth was used to inoculate 36 mL TSB medium supplemented with 1.5 % NaCl. These cultures were again incubated for 16 h (16 ± 1°C), after which the cultivated broth was centrifuged (2465 x *g*, 2 x 10 minutes, 16 °C), and the pellet re-suspended in 40 mL autoclaved artificial seawater. Bacterial titers were verified by making a tenfold dilution series in triplicate on TSA plates supplemented with 1.5 % NaCl, prior to administration.

4.3.3 Experimental design

Following anesthesia using tricaine methanosulfonate (MS-222; 100 mg L^{-1}; Sigma Aldrich N.V., Belgium), a tag (T-bar anchor tag, Floy Tag Inc., USA) was inserted in the caudal epaxial musculature of all fish. The weight of the fish (W_B) was registered.

On both the P and NP side, three distinct square 'treatment zones' (± 2.3 cm^2) were defined, with an interspace of 0.5 cm (Figure 4.1). In one zone, the scales and overlying epidermis were removed by scraping with a scalpel (mechanical treatment, MT). In a second zone, the mucus layer on top of the epidermis was removed using 80 % ethanol (chemical treatment, CT) (Wong *et al.*, 1986; Kanter *et al.*, 2006). The third zone was the non-treatment zone (NT) where the skin was left intact and therefore served as the control zone. The sequence of all treatments was altered on each fish as follows: (subgroup 1) MT – CT – NT; (2) MT – NT – CT; (3) NT – MT – CT; (4) NT – CT – MT; (5) CT – NT – MT; (6) CT – MT – NT, resulting in six subgroups (1 - 6) of 10 fish each (Figure 4.1).

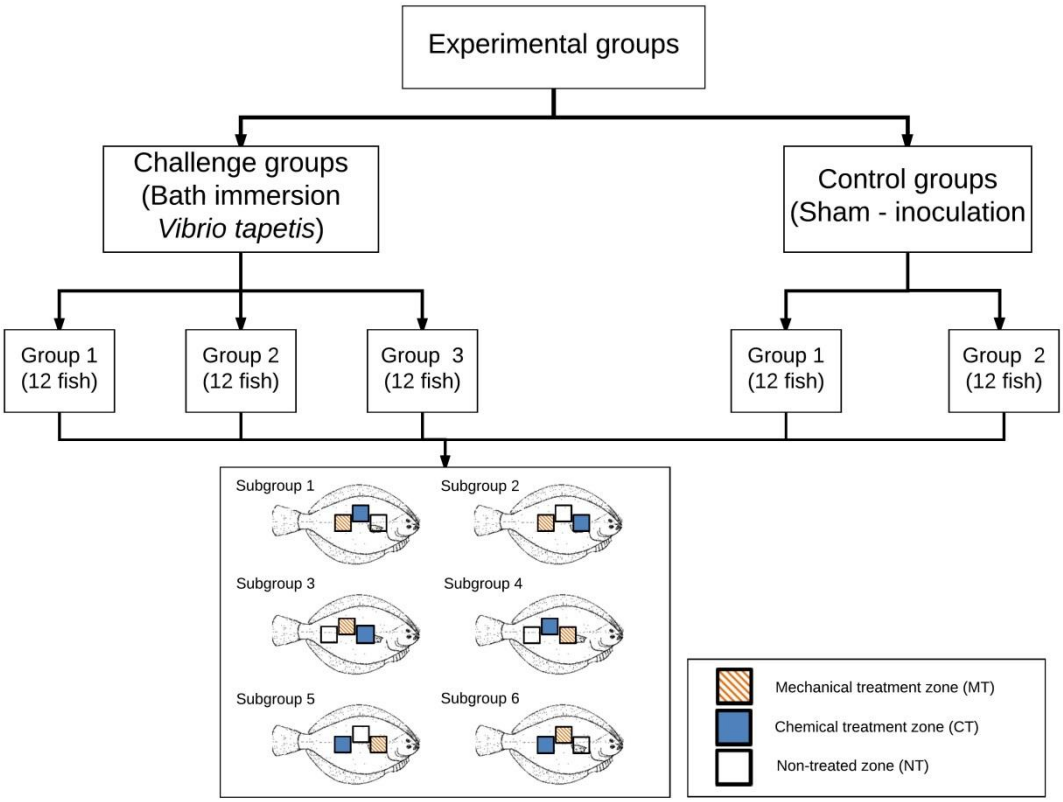

Figure 4.1: Schematic overview of the experimental design. On both pigmented and non-pigmented sides, three distinct treatment zones were defined; a mechanical, chemical and non-treatment zone. The sequence of all treatments was altered on each fish, resulting in six subgroups of each ten fish.

Following recovery, all fish were divided into two groups; fish which were to be challenged with *V. tapetis* and a sham-treated control group, including three and two tank replicates (henceforth referred to as replicates), respectively. Each replicate contained two fish of each subgroup (1 - 6: see above) hence a total of 12 fish which were placed in a separate tank containing 35 L artificial seawater (55 x 38 x 28 cm) (Figure 4.1). The bacterial suspension of *V. tapetis* was added to the water of the three tank replicates resulting in a final concentration of 3.3×10^5 colony forming units (CFU) mL^{-1} (challenge group, n = 36). The fish of the two tank replicates constituting the control group (n = 24) were subjected to the same procedures as the challenge group, but without addition of the bacterial suspension. After 60 minutes, the fish of each replicate were transferred to the 450 L tank after which clinical signs and mortality were recorded on a daily basis during 21 days post-inoculation (DPI). Every second day, starting 5 DPI, all fish were netted, clinically inspected and the P and NP side of each fish photographed to determine the "gross ulceration score" (GUS), "total affected area" (TA) and "ulcerative area" (UA) (see further). As soon as the humane endpoints (extensive lesions and/or subcutaneous hemorrhages, weak or no reaction upon handling) were observed, the fish was euthanized using an overdose of MS-222 (500 mg L^{-1}) and necropsied as described below. At 21 DPI, all surviving fish were sacrificed by an overdose of MS-222 and necropsied as outlined further.

4.3.4 Post-mortem examination

All fish were measured (standard length, L) and weighed (W_E) to calculate the Fulton condition factor (Fulton, 1904) ($K_E = 100 * (W_E / L^3)$) enabling a comparison with the body condition of the fish before the treatment ($K_B = 100 * (W_B / L^3)$). The sex of the fish was recorded and both sagittal otoliths were collected to determine the age using the method described by Imsland *et al.* (2014). A parasitological examination was performed using wet mount preparations of gill biopsies and skin mucus samples. Fish were photographed on both sides to evaluate the presence and/or type of lesions and to calculate the GUS, TA and UA (see further). Finally, a full necropsy was performed whereby samples of the skin and internal organs were collected for bacteriological and histological examination.

4.3.5 Skin lesion assessment

Gross ulceration score (GUS)

To quantify the severity of an ulcerative skin lesion that developed in the treatment zones (MT - CT - NT), a scoring system was applied. The "gross ulceration score" (GUS) was defined as the sum of the scores of seven parameters relating to the depth, healing, elevation of the edge of the lesion, pigmentation in or around the lesion, color of the lesion, hemorrhages around the lesion and the shape of the lesion (See Supplementary file 1). Inherently, ulcerations with a higher GUS reflect a more severe lesion. Scoring was conducted blindly using photographs of all fish collected every second day, starting at 5 DPI, and on the day the fish died.

Total affected area (TA) and ulcerative area (UA)

To quantify the extent of the ulceration, the area of the fish's skin affected by the lesion was determined. The "Total affected area" (TA) of each treatment zone (MT – CT – NT) was calculated using scientific image analysis software (ImageJ 1.4) and represents the total skin area affected by the ulceration, including the healing edge and/or hemorrhages around the lesion. The surface of the open, active lesion is named the "Ulcerative area" (UA).

4.3.6 Bacteriological examination

Skin samples of all treatment zones (MT – CT – NT), as well as samples of liver, kidney and spleen, were inoculated on thiosulfate citrate bile salt sucrose agar (TCBS; Sigma Aldrich N.V., Belgium) and incubated for three days at 16 ± 1 °C. *V. tapetis* was identified by a PCR-based method using REP1D and REP2D sequences [27].

4.3.7 Histological examination

Tissue samples of all skin treatment zones (MT – CT – NT), as well as samples of gill, liver, spleen, intestine, kidney and heart were fixated for 24 h in a phosphate-buffered 4 % formaldehyde solution. Tissues were processed according to standard techniques, sectioned (5 µm) and stained with hematoxylin and eosin (H & E).

4.3.8 Immunohistochemistry

Demonstration of *V. tapetis* in a subset of skin samples (at least 20 % of the fish per group) by means of immunohistochemistry was performed as described previously (Vercauteren *et al.*, 2017). In short, 5 µm tissue sections were incubated with antibodies against *V. tapetis* (1 : 500). Subsequently, bound antibodies were visualized using the DAKO Envision+ System/HRP diaminobenzidine (DAB+) staining kit (DakoCytomation, Glostrüp, Denmark).

4.3.9 Data processing and statistical analysis

To investigate the difference in mortality between the control and challenge group, a logistical regression was used. Fish-specific data namely length, weight, body condition, sex and age gathered during clinical examination was analyzed using a linear mixed model.

To study the role of *V. tapetis* and the effect of the three treatments in the development of skin ulcerations, the total experimental follow up was divided into three distinct periods: 0 - 5 DPI, 6 - 15 DPI and 16 - 21 DPI. For each period, a distinction was made between fish that died before and fish that survived until the end of the experimental period (21 DPI). The fish that died before the end of the experimental period were excluded from the statistical analysis for estimating the differences in GUS, TA and UA. Thus, only the surviving fish were included in the analyses on 5, 15 and 21 DPI. The resulting data of fish that died during the experiment were reported in a descriptive manner. For estimating the difference in GUS, TA and UA, a linear mixed model (proc GLIMMIX) was applied, followed by pairwise comparisons using a Tuckey-Kramer adjustment for multiple testing. GUS, TA and UA were used as response variables. Different treatments (MT – CT – NT), groups, replicates, and subgroups (variable order of the treatments) and side (P or NP) were implemented as co-variables. Analysis on differences between P and NP side was performed in the same manner with side and group as response variables. Interaction effects were studied to estimate group-dependency. In all models, tank was included as a random intercept.

Statistical results were considered to be significant when p-values were lower than 0.05. A p-value between 0.05 and 0.1 was considered as a trend. All statistical analyses were performed using SAS 9.4 and graphs were constructed using R Studio.

4.4 Results

The main fish characteristics are listed in Table 4.1. The mean length of the fish was 21.7 ± 2.8 cm in the challenge and 22.8 ± 3.0 cm in the control group. In total, 21 males and 39 females were included with 16 males and 20 females and 5 males and 19 females in the challenge and the control group, respectively. The mean age of fish included in the challenge group (35.4 ± 12.6 months) was slightly lower, albeit not significantly, compared to the age of the control group (37.4 ± 12.0 months) ($p = 0.6703$). The fish from the challenge ($K_B = 0.99 \pm 0.1$) and control ($K_B = 1.0 \pm 0.2$) group displayed a similar body condition at the beginning of the study (K_B; $p = 0.3958$) remaining stable throughout the total experimental period (K_B - K_E) with no difference between challenged and control fish ($p = 0.6144$).

In one fish of the control group, a parasite resembling *Lernanthropus* spp. was noted on the gills. In two fish of the challenge group, a parasite with a morphology similar to *Tetrahymena* spp. was found on the skin mucus albeit in low numbers.

Length ($p = 0.2190$), weight (W_B: $p = 0.1635$; W_E: $p = 0.2191$), body condition (K_B - K_E: $p = 0.6144$) and age ($p = 0.6703$) of fish included in the control group showed no significant differences with fish from the challenge group and were therefore not implemented in further analyses. Based on a first descriptive analysis, no difference was observed between males and females regarding skin ulceration development. Additionally, only 3 fish on a total of 60 fish harboured parasites in the gills or on the skin and these fish did not develop more severe ulcerations. Therefore, sex and parasitic load were were not implemented in further analyses.

A significantly higher mortality was found in the challenge group (55.6 %) compared to the control group (16.7 %) ($p = 0.0060$). The main mortality peak was encountered on 4 DPI whereby 12 out of 36 challenged fish died.

No macroscopic abnormalities were seen in the gills and internal organs from the fish of the challenge or the control group upon necropsy. These findings were confirmed by histological examination.

4.4.1 Chronological changes in skin lesions following challenge with *V. tapetis*

Non-treated (NT-) zone

No ulcerations developed neither in the challenge nor in the control group. *V. tapetis* was isolated from the NT-zone in two challenged and two control fish at 21 DPI.

Chemical treatment (CT-) zone

In the control group, nine out of the 24 fish developed skin ulcerations in the CT-zone. One of these fish died at 4 DPI with a severe ulceration with a GUS of 9, TA of 3.1 cm² and UA of 3.2 cm². In two out of eight surviving fish, the ulceration had healed at 21 DPI, the other six fish showed signs of healing. In the challenge group, 16 out of the 36 fish developed skin ulcerations in the CT-zone. Nine and three fish died before 5 and 15 DPI, respectively. The average GUS, TA and UA scores of the CT-zone ulcerations are listed in Table 4.2.

The difference between the GUS, TA and UA of the ulcerations in the CT-zone of surviving fish were not statistically significant between the control and challenged fish throughout the entire experimental period, based on the analyses at 5 DPI (GUS: $p = 0.0729$; TA: $p = 0.6443$; UA: p =

Table 4.1: Main fish characteristics (length, weight, body condition, sex and age) per tank replicate and per group as well as mortality.

Group	Replicate	No.	Length (cm)	Weight (g)		Body condition		Sex	Age (months)	Mortality		
				W_B	W_E	K_B	K_E			<5 DPI	<15 DPI	<21 DPI
Challenge	1	12	22.6 ± 3.4	119.8 ± 51.7	116.5 ± 50.2	1.0 ±0.1	1.0 ± 0.1	5 M / 7 F	41.0 ± 14.8	2	1	0
	2	12	20.6 ± 2.2	86.6 ± 26.6	85.9 ± 30.4	1.0 ±0.1	0.9 ± 0.1	6 M / 6 F	29.7 ± 5.6	6	2	0
	3	12	21.7 ± 2.8	105.7 ± 39.3	106.3 ± 39.3	1.0 ±0.1	1.0 ± 0.1	5 M / 7 F	35.0 ± 13.4	8	1	0
	Mean		**21.7 ± 2.9**	**104.0 ± 41.7**	**103.8 ± 42.1**	**1.0 ±0.1**	**1.0 ± 0.1**	**16 M / 20 F**	**35.4 ± 12.6**			
Control	1	12	22.8 ± 2.9	124.3 ± 56.4	120.1 ± 54.6	1.0 ± 0.2	1.0 ± 0.1	2 M / 10 F	38.5 ± 7.8	0	0	1
	2	12	22.9 ± 3.2	130.2 ± 58.3	128.8 ± 61.1	1.0 ± 0.1	1.0 ± 0.1	3 M / 9 F	36.3 ± 15.5	2	0	1
	Mean		**22.8 ± 3.0**	**127.3 ± 56.2**	**124.3 ± 56.7**	**1.0 ± 0.2**	**1.0 ± 0.1**	**5 M / 19 F**	**37.4 ± 12.0**			

W_B: Weight at the beginning of the experimental period; W_E: Weight at the end of the experimental period; K_B: Fulton condition index at the beginning of the experimental period; K_E: Fulton condition index at the end of the experimental period; M: male fish; F: female fish.

0.6933), 15 DPI (GUS: p = 0.7525; TA: p = 0.7976; UA: p = 0.9136) and 21 DPI (GUS: p = 0.9968; TA: p = 1.0000; UA: p = 0.9814).

Mechanical treatment (MT-) zone

The GUS, TA and UA scores of MT-zone ulcerations are listed in Table 4.3.

0 - 5 DPI

Two fish of the control group died at 4 DPI. One fish showed small ulcerations with a GUS of 6.5 ± 2.1 and TA of 0.9 ± 0.7 cm². The other fish was euthanized as it showed extensive hemorrhages and large and severe ulcerations. These ulcerations had a GUS of 8.5 ± 2.1 and TA of 4.3 ± 2.3 cm². *V. tapetis* was not isolated from any fish. Immunohistochemical analysis of a skin ulceration of the second fish on the P side revealed small numbers of comma-shaped immunopositive cells at the edge of the ulceration on top of the stratum spongiosum (dermis) not invading the muscular tissue.

Merely all fish of the control group (n = 22) that were alive at the end of the first experimental period showed ulcerations with a GUS of 5.4 ± 4.0. The TA of the ulcerations was 1.1 ± 1.3 cm² whereby 0.2 ± 0.3 cm² was observed to be active (UA).

In the challenge group, 16 fish died before 5 DPI, mainly on 4 DPI (Table 4.1). The observed skin ulcerations in these fish had a GUS of 9.3 ± 2.3, with a TA and UA of 2.1 ± 1.2 cm² and 1.5 ± 1.3 cm², respectively. From three of five sampled fish, *V. tapetis* was isolated from the ulceration. One of the fish that died at 5 DPI harbored a pure culture of *V. tapetis* in the spleen. Immunohistochemical analyses of the ulcerations of eight sampled fish revealed the presence of comma-shaped immunopositive cells. The cells were located at the ulcerated surface and in underlying dermal tissue. In all cases, the immunopositive cells infiltrated into the muscular tissue superficially, with occasional observations of the cells in the muscular connective tissue (Figure 4.2).

At 5 DPI, the remaining individuals of the challenge group (n = 20) all showed ulcerations with a GUS of 8.5 ± 2.3. The ulcerations had a TA of 1.5 ± 0.9 cm² and UA of 1.1 ± 0.9 cm².

The GUS ($p = 0.0035$) and UA ($p < 0.0001$) of the surviving fish at 5 DPI were significantly higher in the challenge group compared to the control group (Figure 4.3a and 4.3c). The TA did not show a significant difference between challenge and control groups ($p = 0.6682$) (Figure 4.3b).

6 - 15 DPI

None of the control fish died. The GUS at 15 DPI was 4.4 ± 3.5. TA and UA of the ulcerations 0.8 ± 0.8 cm² and 0.1 ± 0.2 cm², respectively.

In the challenge group, four out of 20 remaining fish died between 6 and 15 DPI. Ulcerations were found in all fish (GUS 8.0 ± 3.5). The TA and UA were 1.7 ± 0.9 cm² and 1.0 ± 0.8 cm², respectively. In two of the four fish, *V. tapetis* was isolated from the liver. *V. tapetis* was not retrieved from the ulcerations on the MT-zone. In two of the four fish, comma-shaped immunopositive cells were visualized in the skin ulceration using immunohistochemical staining.

The remaining challenged fish (n = 16) all showed ulcerations with GUS, TA and UA values of 7.0 ± 2.6, 1.2 ± 0.9 cm² and 0.4 ± 0.6 cm², respectively.

At 15 DPI, the GUS of the ulcerations of the challenge group was significantly higher compared to the control group ($p < 0.0001$) (Figure 4.3a). A statistically significant difference was noted for the TA and UA between the challenge and control group ($p < 0.0001$ and $p = 0.0002$, respectively) (Figure 4.3b and 4.3c).

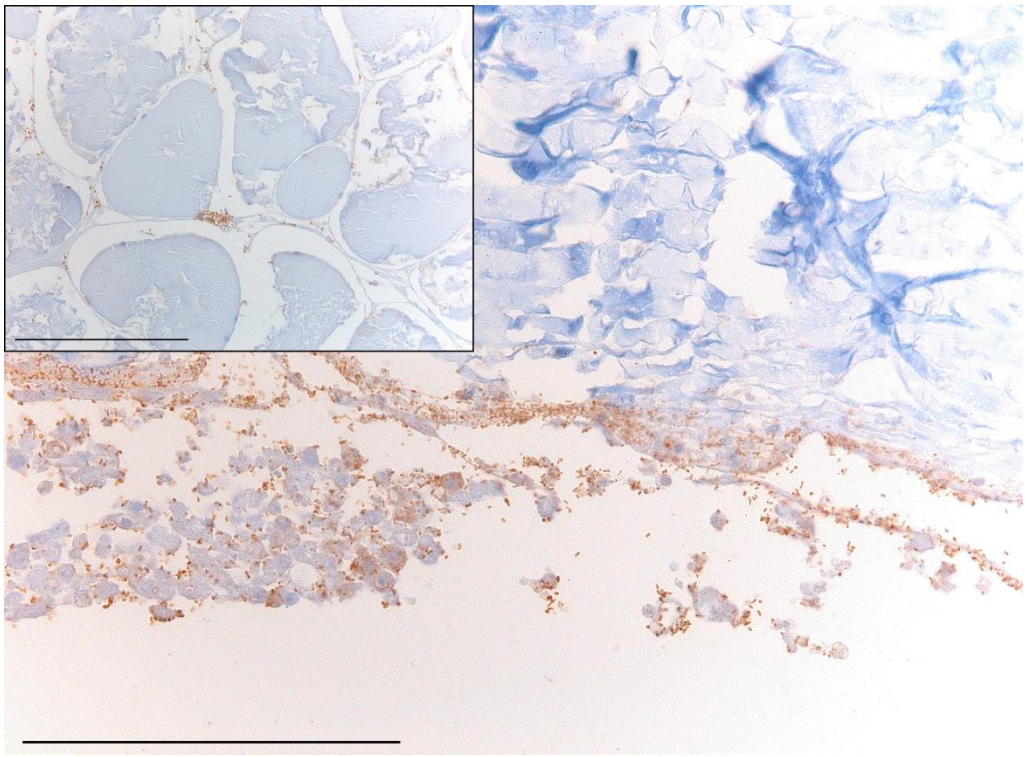

Figure 4.2: Immunohistochemical results demonstrating the presence and location of comma-shaped immunopositive cells (brown). Example of a skin ulceration of a challenged fish that died at 4 days post inoculation. In the exposed dermal tissue and interstitial space of the underlying muscular tissue comma-shaped immunopositive cells are noted. The insert shows a higher magnification to point out the invasion of the immunopositive cells in the interstitial spaces of the muscle. Scale bar = 100µm.

16 - 21 DPI

Between 16 and 21 DPI, two control fish died. The ulcerations in the MT-zone had a GUS of 3.8 ± 3.3, TA of 0.6 ± 0.6 cm² and UA of 0.1 ± 0.1 cm². *V. tapetis* was not isolated and immunohistochemical analysis did not reveal immunopositive cells in the ulcerations.

The remaining control fish (n = 20) were sacrificed at 21 DPI, of which 14 and six fish had ulcerations on both sides or only on the P side, respectively. The ulcerations had a GUS of 4.5 ± 2.8. The TA was 0.7 ± 0.7 cm² whereby 0.1 ± 0.2 cm² was observed to be active (UA). From one fish, *V. tapetis* was isolated from an ulceration in the MT-zone. This was not confirmed by immunohistochemical staining.

None of the 16 remaining challenged fish died. At 21 DPI, the ulcerations found in the sacrificed fish had a GUS, TA and UA of 6.0 ± 2.8, 1.1 ± 0.7 cm² and 0.3 ± 0.5 cm², respectively. *V. tapetis* was isolated from the ulcerations of four fish. The immunohistochemical analysis did not reveal immunopositive cells in the ulcerations of any of the sampled fish.

At 21 DPI, a trend of a higher GUS of ulcerations of challenged compared to control fish (p = 0.0600) was noted (Figure 4.3a). The TA (p = 0.0030) and UA (p = 0.0006) remained significantly higher in the MT-zone ulcerations of the challenge group compared to the control group (Figure 4.3b and 4.3c).

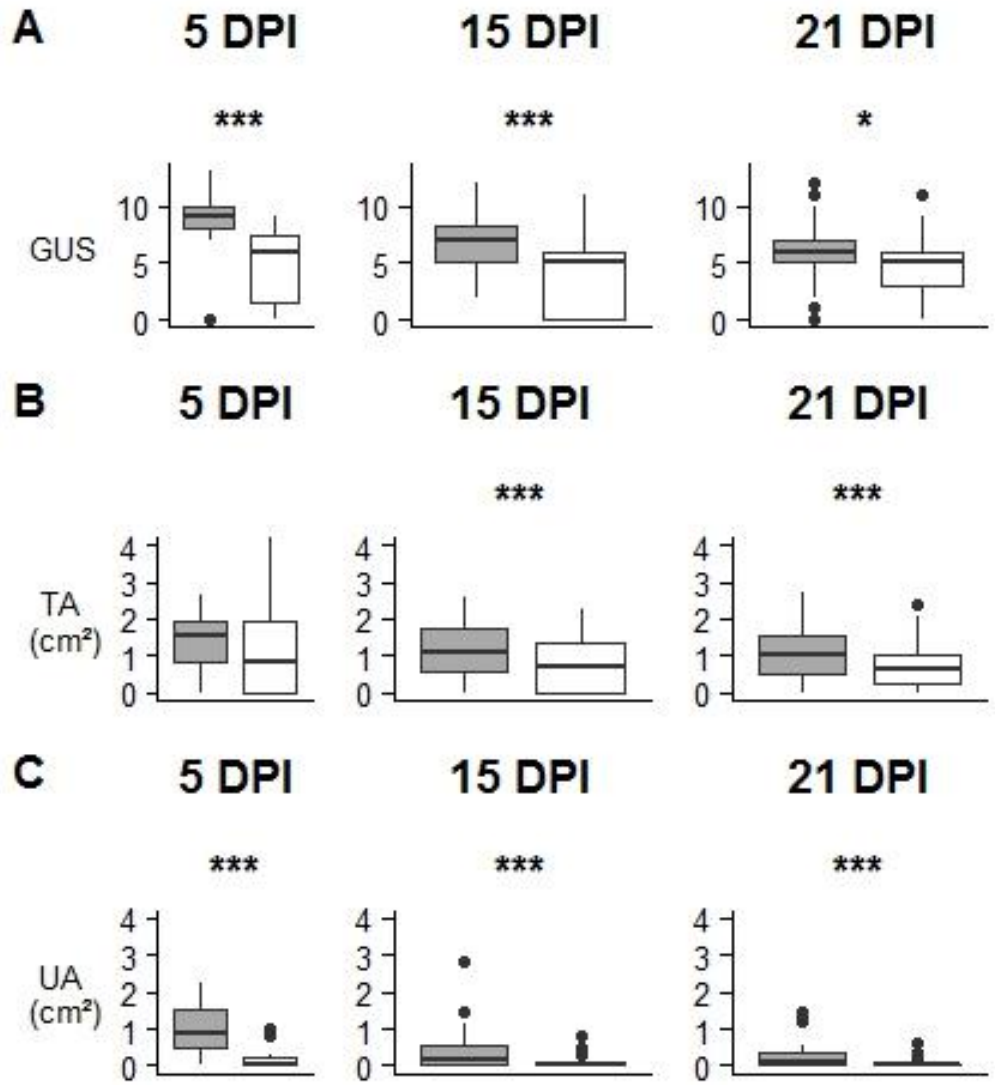

Figure 4.3: Overview of skin lesion assessment for mechanically treated zones during the experimental period. Comparison of the main parameters between challenge (grey) and control (white) groups at 5, 15 and 21 days post inoculation (DPI). Only the fish that were alive at the end of the experimental period (21 DPI) are depicted. Three asterisks (***) indicate a significant difference between challenge and control group, one asterisk (*) represents a trend. (A) Gross ulcerations score (GUS) were significantly higher in challenge group at 5 and 15 DPI. At 21 DPI the same trend was visible. (B) Total affected area (TA) was higher in challenge group compared to the control group at 15 and 21 DPI. (C) Ulcerative area (UA) was higher in challenge group compared to the control group during the entire experimental period.

The average GUS, TA and UA scores of the ulcerations in the MT-zones were significantly higher compared to the CT-zones at 5 DPI (GUS: p = 0.0058; TA: p = 0.0097; UA: p = 0.0353), 15 DPI (GUS: p = 0.0035; TA: p = 0.0029; UA: p = 0.0410) and 21 DPI (GUS: p = 0.0026; TA: p = 0.0028; UA: p = 0.0333). These results were independent from the group (control or challenge).

Histological examination confirmed the gross appearance of the lesions. Ulcerations were characterized by focal loss of epidermal and/or dermal tissue. The dermis was often infiltrated by mild to moderate amounts of inflammatory cells. Underlying muscle tissue was regularly degenerated and/or infiltrated by inflammatory cells. Partially healed ulcerations were mainly typified by a one or two cell-layered epidermis overlying a disrupted dermal tissue without the presence of scales and/or scale pockets. Inflammatory cells were present in moderate amounts and were localized mainly in dermal and muscular tissue. Hemorrhages were rather moderate. These observations were similar in the challenge and the control group and in lesions occurring on the P and NP side.

4.4.2 Chronological changes in skin lesions on the pigmented and non-pigmented side

Since the main difference between the challenge and control group in the development of skin ulcerations was found in the MT-zones, only these zones are discussed when comparing the ulcerations in the pigmented (P) and non-pigmented (NP) sides of the fish.

At 5, 15 and 21 DPI, the difference between both sides was not dependent on the group the fish belonged to (control or challenge). Therefore, in the statistical analysis, the results of the control and challenged fish were taken together.

0 - 5 DPI

Merely all surviving fish (n = 42) developed ulcerations on both P and NP sides except for four fish with only an ulceration on the P side. The GUS of the ulcerations developed on P and NP side at 5 DPI was 8.6 ± 1.8 and 6.1 ± 4.1, respectively. TA and UA of ulcerations were 2.2 ± 0.9 cm^2 and 1.0 ± 1.1 cm^2 on the P side and 0.6 ± 0.6 cm^2 and 0.5 ± 0.6 cm^2 on the NP side, respectively. Generally, the ulcerations on the P side were significantly more severe (GUS, $p = 0.0321$) and larger (TA, $p = 0.0066$) compared to the NP side (Figure 4.4a and 4.4b). The part of the ulcerations that was active (UA) was similar between the P and NP side ($p = 0.1580$) (Figure 4.4c).

6 - 15 DPI

At 15 DPI, all fish (n = 38) had developed an ulceration on the P side while only 24 fish displayed an ulceration on the NP side. The GUS of the ulceration on the P side was significantly higher (7.5 ± 2.3) compared to the GUS of the ulcerations on the NP side (3.5 ± 3.1) ($p = 0.0058$) (Figure 4.4a). The TA of the ulcerations was 1.6 ± 0.7 cm^2 on the P side whereby 0.4 ± 0.6 cm^2 was found to be active (UA). On the NP side, TA of the ulcerations was 0.3 ± 0.4 cm^2 with UA of 0.1 ± 0.2 cm^2. The TA was significantly higher in P side compared to the NP side ($p = 0.0017$) and the same trend was present upon considering the UA ($p = 0.0538$) (Figure 4.4b and 4.4c).

16 - 21 DPI

Of the surviving fish (n = 36), 29 had developed an ulceration on both sides and 7 fish showed ulcerations only on the P side. The GUS of the ulcerations on the NP side was 3.6 ± 2.6. The TA and UA of the latter were 0.4 ± 0.3 cm^2 and 0.1 ± 0.1 cm^2, respectively. The ulcerations on the P side had a GUS of 6.7 ± 2.1, TA of 1.4 ± 0.6 cm^2 and UA of 0.3 ± 0.5 cm^2. Comparing ulcerations on the P side and NP side, at 21 DPI, ulcerations on the P side were generally more severe ($p = 0.0126$) and had a larger surface (TA; $p = 0.0029$) compared to the ulcerations on the NP side (Figure 4.4). The UA showed a similar trend ($p = 0.0528$).

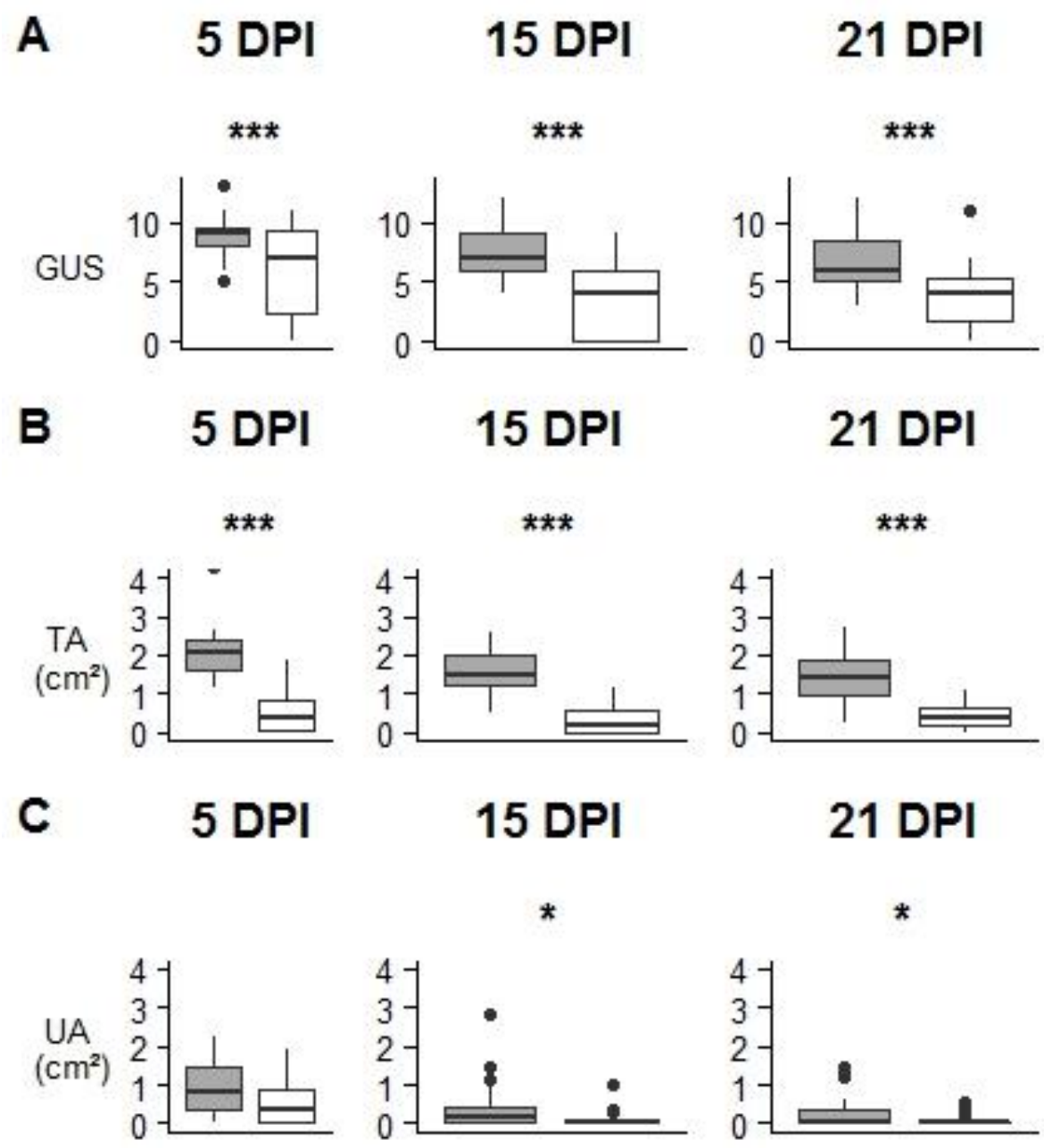

Figure 4.4: Overview of skin lesion assessment for mechanically treated zone ulcerations on pigmented and non-pigmented sides. Comparison of the main parameters between pigmented (grey) and non-pigmented (white) sides at 5, 15 and 21 days post inoculation (DPI). Results of the control and challenge fish were taken together. Only the fish that were alive at the end of the experimental period (21 DPI) are depicted here. Three asterisks (***) indicate a significant difference between pigmented and non-pigmented side, one asterisk (*) represents a trend. (A) Gross ulcerations score (GUS) and (B) Total affected area (TA) were higher on the pigmented side compared to the non-pigmented side during the entire experimental period. (C) Ulcerative area (UA) did not differ significantly although a similar trend was visible at 15 and 21 DPI.

4.5 Discussion

The results of the present study strongly point towards the involvement of *V. tapetis* in skin ulcerations since more common dab inoculated with a bacterial suspension of *V. tapetis* (3.3×10^5 CFU mL^{-1}, an inoculation dose comparable to another study (Bergh & Samuelsen, 2007) developed skin ulcerations compared to control fish that were sham-treated. Furthermore, skin ulcerations were significantly more severe and covered a larger surface in the challenged fish. Following bacteriological analysis, *V. tapetis* was isolated from skin ulcerations of eight fish in total and using immunohistochemical staining, comma-shaped immunopositive cells, presumed to be *V. tapetis* were visualized in these skin ulcerations. Notably, *V. tapetis* was isolated predominantly from the ulcerations of fish that died at 4 DPI. This observation was again supported by immunohistochemical staining where mainly in those fish the comma-shaped immunopositive cells were visualized.

To determine the role of the skin or mucus as a protective barrier against infection and colonization by *V. tapetis*, three treatments were applied on the skin. After inoculation with *V. tapetis*, ulcerations developed predominantly in the mechanically treated zone where scales and overlying epidermis were removed. Moreover, the ulcerations in this zone were more severe compared to ulcerations in chemically treated zone where the mucus was locally removed using ethanol. In the non-treated zone that served as control zones, no skin ulcerations developed.

The mechanical treatment removes the scales and overlying epidermis which leaves the dermal tissue exposed hereby resembling wounds inflicted by predators or fishing gear at sea (Davis & Ottmar, 2006). Exposure of dermal tissue could facilitate the adherence of *V. tapetis* to e.g. exposed fibronectin and collagen. The virulence factor(s) of *V. tapetis* that could possibly be involved in this process are not yet elucidated, although adhesion components such as pili were described in strains pathogenic to mollusks (Paillard & Maes, 1994; Madec *et al.*, 2014). The adhesion may be followed by the further destruction and invasion of the host tissue. Based on immunohistochemical staining in the present study, it was shown that comma-shaped immunopositive cells, presumed to be *V. tapetis,* were able to invade the dermis and adjacent muscular tissue, which is often mediated by several enzymes degrading the extracellular matrix. In *V. tapetis* isolates pathogenic to mollusks, 87 different proteins including phospholipases and proteases were demonstrated in their secretome (Madec *et al.*, 2014). However, at present, no such data are available for *V. tapetis* isolates collected from fish.

The chemical treatment removed the mucus layer, which has various protective properties, both mechanical and immunochemical (Svendsen & Bøgwald, 1997; Raj *et al.*, 2011). The mucus contains various antibacterial proteins such as paradaxin, pleurocidin and parasin 1 and quickly accumulates in a wound region forming a protective layer (Subramanian *et al.*, 2008; Verma *et al.*, 2017). The results of the present study suggest that *V. tapetis* is to a lesser extent hampered by the mucus layer since less fish developed skin ulcerations in the chemical treatment zones. However, it is difficult to make a clear statement since, according to the 'dynamic mucus coat' principle, depletion of the mucus at one site may quickly be compensated and replaced by mucus from adjacent zones (Svendsen & Bøgwald, 1997). This might have resulted in a quick or immediate repair of the mucus layer and resumed protection of the skin against bacterial invasion.

The absence of skin ulcerations in the non-treated zones might indicate that an intact skin indeed provides an adequate barrier against *V. tapetis* infection and/or colonization.

The asymmetry of flatfish has since long been a fascinating topic (Vethaak, 1992; Wiklund, 1994). Although one might expect morphological or even functional differences between both sides, merely a handful of studies investigated this phenomenon. Faílde *et al.* (2014) showed that, besides a difference in pigmentation, a higher number of mucus-producing goblet cells with an occasional observation of clusters of such cells were found on the pigmented compared to the non-pigmented side in turbot (*Psetta maxima*). In the current study, more, severe and larger ulcerations developed on the pigmented side indicating a higher susceptibility. In contrast, Vethaak (2013) reported higher incidences of skin ulcerations on the non-pigmented side in wild-caught flounder (*Platichthys flesus*). Wiklund and Bylund (1993) reported a sex-related distribution of ulcerations on pigmented and non-pigmented side of wild-caught flounder. The reason(s) for these apparently divergent findings hitherto remain(s) obscure.

It should be kept in mind that in general, skin ulcerations in fish are considered to have a multifactorial cause (Mellergaard & Nielsen, 1997). Therefore, it may be assumed that other factors, such as fish-related characteristics (age, sex, immunity), can influence the development of skin ulcerations. Sex is frequently pointed out in literature as a predisposing factor for skin ulcerations (Wiklund & Bylund, 1993; Mellergaard & Nielsen, 1997; Lang *et al.*, 1999). The current study does not allow drawing conclusions on a possible difference in susceptibility between males and females due to the low amount of male fish.

Questions may rightfully arise on the impact of these ulcerations on the health and survival of the fish. In literature, effects such as loss of appetite (Vilar *et al.*, 2012), loss of reflexes (Davis & Ottmar, 2006), osmotic imbalance (Mellergaard and Nielsen, 1997) and changing swimming behavior (Vilar *et al.*, 2012) are mentioned. Based on the information gathered in this study, one may speculate that the infection with *V. tapetis* and subsequent development of skin ulcerations impacts the survival of the fish as a peak in mortality of challenged fish was observed at 4 DPI, and overall, the mortality in challenged fish (55.6 %) was significantly higher compared to control fish (16.7 %). Septicemia caused by *V. tapetis* was already reported in corkwing wrasse and Atlantic halibut (Jensen *et al.*, 2003; Reid *et al.*, 2003). Nevertheless, in the study of Lopez *et al.* (2011), *V. tapetis* was only isolated from ulcerations and not from internal organs of wedge sole. In our study, a monoculture of *V. tapetis* was isolated from the spleen or liver of three fish that had died. However, since only a small number of bacterial isolates could be gathered of the dead fish due to post-mortem decay, the results were inconclusive. Beside septicemia, the engendered osmotic imbalance might also have induced mortality. Indeed, the skin is an important organ for maintaining the osmotic balance, whereby damage, as observed in skin ulcerations might cause a shift from an osmotic balance to an imbalance, leading to death (Carlisle & Roberts, 1977). Damage covering as little as 10 % of the body surface area was reported to cause high mortalities (Noga, 2000). This reasoning together with the finding that the skin ulcerations in the fish that died were generally more severe than those in surviving fish, contribute to rectifying the hypothesis that the observed mortalities were due to osmotic imbalance. Blood analysis in future experiments might corroborate this reasoning (Järvi, 1990; Komoroske *et al.*, 2016).

In the present study, wild-caught common dab was included contributing to the representativeness of the obtained results in the field. Nevertheless, working with wild-caught fish inevitably entails that no information on the history or characteristics of the fish is available which most likely will increase the inter-individual variability and enhance the possibility for unforeseen complications. The latter greatly applies to the presence of (non-)infectious diseases. In this study, eight fish were included in the experiment with pre-existing small ulcerations (n = 4) or multifocal bulging lesions (1 - 2 mm) (n = 4) (data not shown). Furthermore, five other fish developed similar small bulging lesions during the

experimental period. In a descriptive evaluation, mortality nor development of lesions were different in fish with pre-existing lesions compared to the others. Therefore, this parameter was not included in further analysis. In addition, a mortality of 16.7 % was noted in the control group for which no cause was established. The same observation was made when wild corkwing wrasse was kept in captivity with typically 1 - 5 % daily mortality in the population starting 1 month after capture (Bergh & Samuelsen, 2007). From fish both from the challenge and control group, *V. tapetis* was recovered before the trial was initiated (data not shown) and following necropsy during the experiment. These findings point towards the facultative pathogenic nature of *V. tapetis* which is in agreement with a report on *V. tapetis* as opportunistically pathogenic to corkwing wrasse (Bergh & Samuelsen, 2007). This again rectifies the findings in the present study that prior skin damage acts as a major contributing factor to the development of skin ulcerations.

In conclusion, *Vibrio tapetis* is able to cause skin ulcerations although a breach of the skin barrier seems to be a major contributing factor to the development of skin ulcerations. The pinpointed infection model using bath immersion may be used in the future to study the role of various anthropogenic or environmental factors on the development of skin ulcerations.

4.6 Acknowledgements

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Table 4.2: Mean gross ulceration score (GUS), total affected area (TA, cm²) and ulcerative area (UA, cm²) of the ulcerations in the chemically treatment zones in challenged and control fish. Values are subdivided according to period with 0 - 5, 6 - 15 and 16 - 21 days post inoculation (DPI). A subdivision was made between fish that died during the experimental period (D) and fish that survived in that period (S).

Group	Dead or surviving	0 - 5 DPI			6 - 15 DPI			16 - 21 DPI		
		GUS	TA	UA	GUS	TA	UA	GUS	TA	UA
Challenge	D	4.5 ± 5.2	1.6 ± 2.9	1.6 ± 2.9	NA	NA	NA	NA	NA	NA
Control	D	7.8 ± 2.6	1.5 ± 0.9	1.1 ± 1.2	6.7 ± 0.6	1.7 ± 0.5	1.3 ± 1.1	NA	NA	NA
Challenge	S	7.0 ± 1.0	0.8 ± 0.4	0.6 ± 0.6	7.8 ± 4.9	0.7 ± 0.6	0.4 ± 0.7	5.6 ± 1.1	0.4 ± 0.3	0.1 ± 0.2
Control	S	8.4 ± 5.0	0.9 ± 0.6	0.7 ± 0.7	6.5 ± 2.5	0.3 ± 0.2	0.2 ± 0.1	4.3 ± 2.5	0.3 ± 0.2	0.1 ± 0.3

NA: Not available

Table 4.3: Mean gross ulceration score (GUS), total affected area (TA) and ulcerative area (UA) of the ulcerations in the mechanically treatment zones in challenged and control fish. Values are subdivided according to period with 0 - 5, 6 - 15 and 16 - 21 days post inoculation (DPI). A subdivision was made between fish that died during the experimental period (D) and fish that survived in that period (S).

Group	Dead or surviving	0 - 5 DPI			6 - 15 DPI			16 - 21 DPI		
		GUS	TA (cm²)	UA (cm²)	GUS	TA (cm²)	UA (cm²)	GUS	TA (cm²)	UA (cm²)
Challenge	D	9.3 ± 2.3	2.1 ± 1.2	1.5 ± 1.3	8.0 ± 3.6	1.7 ± 0.9	1.0 ± 0.8	NA	NA	NA
Control	D	7.5 ± 2.1	2.6 ± 2.4	1.8 ± 2.8	NA	NA	NA	3.8 ± 3.3	0.6 ± 0.6	0.1 ± 0.1
Challenge	S	8.5 ± 2.3	1.5 ± 0.9	1.1 ± 0.9	7.0 ± 2.6	1.2 ± 0.9	0.4 ± 0.6	6.0 ± 2.8	1.1 ± 0.7	0.3 ± 0.5
Control	S	5.4 ± 4.0	1.1 ± 1.3	0.2 ± 0.3	4.4 ± 3.5	0.8 ± 0.8	0.1 ± 0.2	4.5 ± 2.8	0.7 ± 0.7	0.1 ± 0.2

NA: not available

Chapter 5

Assessing the virulence of *Vibrio tapetis*

Based on: Vercauteren, M. / Rahmani, A., Vranckx, K., Boyen, F., Bidault, A., Pichereau, V., Decostere, A., Paillard, C., & Chiers, K. (2020). MALDI-TOF MS as a promising tool to assess potential virulence of *Vibrio tapetis* isolates. Accepted with revisions in Aquaculture, May 2020.

5.1 Abstract

Vibrio tapetis is the etiological agent of Brown Ring Disease (BRD) mainly affecting the Manila clam (*Ruditapes philippinarum*). Although this bacterium has been mainly known as a clam pathogen, it has been isolated from several fish species. The main aim of the present study was to further explore the variability of 27 *V. tapetis* isolates from bivalves and fish, considering three different aspects; *in vitro* virulence based on the loss of clam hemocyte adhesion properties, detection of the *virB4* gene, which encodes for an essential component of the Type IV Secretion System, and MALDI-TOF MS characterization. Finally, these approaches were compared and evaluated for their ability to discriminate the potential pathogenicity of the 27 isolates against the Manila clams. Among the 11 isolates from the common dab (*Limanda limanda*) isolated in 2018 in Belgium, only one *V. tapetis* isolate (2BB) showed intermediate virulence, against the Manila clam, according to *in vitro* virulence experiments. Among these 11 isolates, seven carry the *virB4* gene while none of the fish isolates tested in previous studies showed the presence of this particular gene. Finally, the peak protein profiles generated with MALDI-TOF MS analysis from all 27 *V. tapetis* strains showed a clear clustering of clam pathogenic and nonpathogenic isolates. Therefore, we can assume that a new isolate of *V. tapetis* that would cluster within the clam pathogenic isolates could be potentially pathogenic to the Manila clam, but *in vivo* BRD reproduction assay must be performed to confirm this purpose. Based on these results, MALDI-TOF MS typing is proposed as a promising tool to discriminate isolates of *V. tapetis* according to their virulence abilities against the Manila clam. This approach allows rapid and cost-efficient identification of *V. tapetis* isolates and opens new perspectives to study the virulence of *V. tapetis* isolates but also to perform environmental monitoring in order to prevent outbreaks.

5.2 Introduction

Vibrio tapetis is the etiological agent of Brown Ring Disease (BRD), mainly affecting Manila clam (*Ruditapes philippinarum*), and responsible for mass mortalities in cultured clams (Maes & Paillard, 1992; Paillard *et al.*, 1994). BRD is characterized by a brown organic, conchiolin deposit, between the pallial line and the edge of the shell (Paillard & Maes, 1995). The reference strain *V. tapetis* CECT4600, first isolated from a cultured Manila clam exhibiting BRD in France, has been well characterized (Paillard & Maes, 1990b; Borrego *et al.*, 1996). Since 1990, *V. tapetis* was identified in BRD of various bivalve hosts such as common cockle (*Cerastoderma edule*) (Novoa *et al.*, 1998; Paillard & Maes, 1990b), rayed artemis (*Dosinia exoleta*) (Paillard, 2004b), pink clam (*Polititapes rhomboids*) (Paillard, 2004b), grooved carpet shell (*R. decussatus*) (Maes & Paillard, 1992; Novoa *et al.*, 1998) and *Venerupis aurea* (Maes & Paillard, 1992). This suggests that *V. tapetis* is able to cross species barriers (Paillard, 2016).

Since 2003, some studies described the isolation of *V. tapetis* from cultivated or captive held aquatic vertebrates such as corkwing wrasse (*Symphodus melops*) (Jensen *et al.*, 2003), Atlantic halibut (*Hippoglossus hippoglossus*) (Reid *et al.*, 2003), Dover sole (*Solea solea*) (Declercq *et al.*, 2015), fine flounder (*Paralichthys adspersus*) and red conger eel (*Genypterus chilensis*) (Levican *et al.*, 2017). *V. tapetis* was also pinpointed as causative agent of ulcerative skin lesions in the wild-caught common dab (*Limanda limanda*) (Vercauteren *et al.*, 2019a).

In the past, *V. tapetis* has been described as a homogenous taxon based on traditional methods such as bio- or serotyping (Castro *et al.*, 1996; Figueras *et al.*, 1996). Later studies, using advanced genetic or experimental techniques revealed that this species is more heterogeneous. To date, three distinct subspecies of *V. tapetis* are described, i.e. subsp. *tapetis* (Balboa & Romalde, 2013), subsp. *britannicus* (Balboa & Romalde, 2013) and subsp. *quintayensis* (Levican *et al.*, 2017). However, using genotyping methods, such as Randomly Amplified Polymorphic DNA (RAPD) PCR (Romalde *et al.*, 2002) and multilocus sequence analysis (MLSA) (Gulla *et al.*, 2017) different clusters were defined between *V. tapetis* subspecies. This indicates that the phylogeny of *V. tapetis* is not yet completely unraveled.

From an epidemical point of view, differences between *V. tapetis* isolates might provide valuable information regarding the diagnosis of BRD outbreaks and detection of virulent isolates. Furthermore, typing of isolates can also help examining the geographical and host distributions supporting a more ecological approach for studying host-pathogen-environment interactions (Paillard, 2016; Rodríguez *et al.*, 2006).

Several studies have indeed pointed towards differences between clam pathogenic and non-pathogenic *V. tapetis* strains. *In vivo* infection into the pallial cavity of the clams revealed that none of the tested strains derived from fish are able to induce BRD in manila clam (Choquet, 2004; Bidault *et al.*, 2015; Dias *et al.*, 2018). In addition, these last authors have pointed out that *virB4*, an essential gene coding for nucleoside triphosphatase of the Type IV Secretion System (T4SS), was only present in strains pathogenic to the Manila clam and therefore suggested to be a discriminative tool to differentiate between pathogenic and non-pathogenic strains (Dias *et al.*, 2018). Furthermore, the *virB4* real-time PCR assay offers a method for quantification of *V. tapetis* in the extrapallial fluids of the Manila clam (Bidault *et al.*, 2015).

The main aim of the present study was to further explore the variability between the 27 *V. tapetis* isolates from bivalves and fish, considering three different aspects; *in vitro* virulence based on the loss of

hemocyte adhesion properties after contact with *V. tapetis* (Choquet *et al.*, 2003), detection of the *virB4* gene using the TaqMan qPCR (Bidault *et al.*, 2015), and protein-based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) characterization. Finally, these approaches were compared and evaluated for their ability to discriminate the potential virulence of the 27 isolates against the Manila clams.

5.3 Materials and methods

5.3.1 Bacterial isolates and cultivation

The *V. tapetis* isolates used in this study were isolated from nine different host species (five bivalves and four fish species) in various countries between 1988 and 2017. Table 5.1 summarizes the information of all isolates with the year, place and host of first isolation and a synthesis of the existing knowledge about their virulence.

For the cytotoxicity bioassay, only the isolates derived from common dab and the CECT4600 reference strain were used. All isolates were cultivated on Tryptic Soy Agar (TSA; DifcoTM) supplemented with 1.5 % NaCl and were allowed to grow for two days at 16 ± 1 °C (fish isolates) or 18 ± 1 °C (reference clam isolates). For the *virB4* gene detection only common dab isolates were used. All isolates were cultured in TSA supplemented with 1.6 % NaCl for PCR and Tryptic Soy Broth (TSB; DifcoTM) supplemented with 1.6 % NaCl for qPCR. For MALDI-TOF analysis, all 27 isolates were cultivated in triplicate (biological replicates) on TSA supplemented with 1.5 % NaCl. Isolates were grown for minimally two days at 16 ± 1 °C (fish isolates) or 18 ± 1 °C (clam isolates).

5.3.2 In vitro hemocyte cytotoxicity bioassay

Virulence of *V. tapetis* isolates derived from common dab was tested using the standardized *in vitro* hemocyte cytotoxicity bioassay (Choquet *et al.*, 2003). This test is based on the ability of *V. tapetis* virulent strains to induce a rounding phenotype on infected hemocytes, increasing the number of non-adherent as compared to the negative control. The type strain CECT4600 was used as the positive control, and filter-sterilized seawater (FSSW) as negative control. Animals used in this study were Manila clams (4cm Manila clam, February 2018) from the SATMAR shellfish aquaculture site in Landeda (Finistère, France). Clams were allowed to acclimate in oxygenated seawater at 14°C for 14 days. Hemolymph was harvested from the adductor muscle, pooled after quality check and the hemocytes were enumerated using a Malassez counting grid. One hundred µl of hemolymph (5×10^5 hemocyte ml^{-1}) was added in 24-well plates and kept for a few minutes to let hemocytes adhere to the bottom of the plate. Then, 100 µl of bacterial suspension prepared with FSSW, was added at a bacteria/hemocyte ratio of 25/1. Hemocyte exposure to each of the bacterial isolates was performed in triplicate. In negative control samples, 100 µl of FSSW was added to the hemolymph. After 3 h of incubation, the number of non-adherent hemocytes was measured (used as a proxy of *V. tapetis* cytotoxicity) using flow cytometry after addition of 4 µl of SYBR–Green solution in DMSO (nucleic acid gel stain, dilution 1 : 10 000, Life Technology, USA) as already described (Choquet *et al.*, 2003). A ratio was calculated by dividing the number of non-adherent hemocytes in samples exposed to bacteria by the number of non-adherent cells in negative controls. Statistical analyses were performed using a pairwise Student test to determine significant differences in non-adherent cell ratios between bacterial suspensions and both positive and negative control samples as already performed by Choquet *et al.* (2003).

5.3.3 VirB4 gene detection

A PCR assay was performed with common dab isolates, in which a fragment of 173 bp of the *virB4* gene was amplified using primers 170513 (5'-TTAAAAGTGGCGGAGGAATG-3') and 170514 (5'-AAGCTCTGCATCGGTTAGGA-3') and GoTAQ polymerase. Subsequently, a Taqman real-time qPCR quantification was performed according to the standardized method previously described (Bidault *et al.*, 2015).

5.3.4 MALDI-TOF MS characterization

An ethanol formic acid extraction was performed, based on MALDI Biotyper protocol (Bruker Daltonics, Bremen Germany). Briefly, bacterial cultures were suspended in nuclease-free water aliquoted in 1.5 ml Eppendorf tubes. Ethanol (70 %) dissolved in high performance liquid chromatography (HPLC) grade water was added to the suspension and tubes were centrifuged twice (20 000 x g, 2 min) upon which the ethanol was removed. Thereafter, 20 µl of 70% formic acid (in HPLC grade water) was added to the pellet. To finish the extraction, 20 µl of acetonitrile was added. One µl of each extract was spotted eight-fold (technical replicates) on a MALDI target plate (Bruker Daltonics, Bremen, Germany), air dried and covered with 1 µl of alpha-cyano-4-hydroxycinnamic acid matrix (Bruker Daltonics, Bremen, Germany). All samples were processed in triplicate (technical replicates) with an Autoflex III Smartbeam MALDI-TOF MS, recording masses ranging from 2 000 to 20 000 Da using standard settings (flexControl 1.4, version 3.4, Bruker Daltonic, Bremen, Germany). The obtained raw spectra were imported in BioNumerics 7.6.3 (Applied Maths NV, Sint-Martens-Latem, Belgium) for data analysis. Preprocessing of the data was performed according to Giacometti *et al.* (2018). After preprocessing, peaks were detected using the continuous wavelet transform method with a signal-to-noise threshold of two. The spectra were summarized in an average spectrum per biological replicate and all replicates with less than 95% similarity to this summary spectrum were removed from the analysis. Of *V. tapetis* 2BB, RD0705 and RP2.3, only two biological replicates were implemented in the analyses due to inconsistencies in the data or low similarity (< 50 %) with other replicates of the same isolate. The resulting summary spectra were used to construct an UPGMA dendrogram using a Pearson similarity coefficient.

5.4 Results and discussion

The wide host and geographic distribution of *V. tapetis* and linked questions on possible variability of isolates have been discussed in previous research with a deep interest in finding a discriminative test for assessing pathogenicity of *V. tapetis* isolates against clams (Bidault *et al.*, 2015). Furthermore, the discovery of new isolates from skin ulcerations in the common dab (Vercauteren *et al.*, 2018) might urge for further exploration of pathogenic markers using available techniques.

5.4.1 Most of the *V. tapetis* isolates from the common dab are unable to induce hemocyte toxicity.

To gain insight into the pathogenicity of *V. tapetis* to the Manila clam, an *in vitro* cytotoxicity assay was developed based on the cell-rounding and subsequent loss of adherence of hemocytes following exposure to *V. tapetis* (Choquet *et al.*, 2003). This bioassay was previously performed for many *V. tapetis* strains pathogenic to bivalves (Table 5.1), whereby strains IS9 and CECT4600 induced the highest loss of adhesion of the hemocytes (i.e. *in vitro* cytotoxicity). Most of the other strains from clams displayed intermediate cytotoxicity and only a few showed no cytotoxicity (Table 5.1). Remarkably, the GDE and GTR-I strains, both isolated from bivalves but not from the Manila clam, showed no cytotoxicity *in vitro*. Complete genome analyses of these two strains revealed strong differences with clam isolates (Dias *et al.*, 2018) and these two strains were genetically closer to the LP2 strain derived from corkwing wrasse.

This result further substantiates the observed difference in cytotoxicity towards Manila clam hemocytes (Dias *et al.*, 2018).

The common dab isolates, 2BG, 2AE, 2AC and 2BW tested in this study, showed non-adherent cells ratios after bacterial exposure fluctuating around 1. The 2BU isolate displayed a ratio of 0.85 ± 0.14 and remained clearly below 1 in all replicates. The ratios found with isolates 2BC, 2BT, 2AU, and 2BA ranged between 1 and 1.5. All isolates showed similar ratios with the FSSW control, therefore indicating negative cytotoxicity (i.e. causing no additional loss of adherence of hemocytes) (p-value > 0.05; Figure 5.1). The isolate 2BB showed the highest cytotoxic activity with an average ratio of 2.27 ± 0.26 (Figure 5.1). The cytotoxicity of 2BB was evaluated to be intermediate since the amount of non-adherent hemocytes was significantly higher as compared to the FSSW control (p-value = 0.0005) but was intermediate considering the positive control strain (p-value = 0.31) These results are consistent with previous ones obtained with strains isolated from fish (e.g. LP2 and HH6087) which mostly showed negative cytotoxicity towards clam hemocytes (Dias *et al.*, 2018).

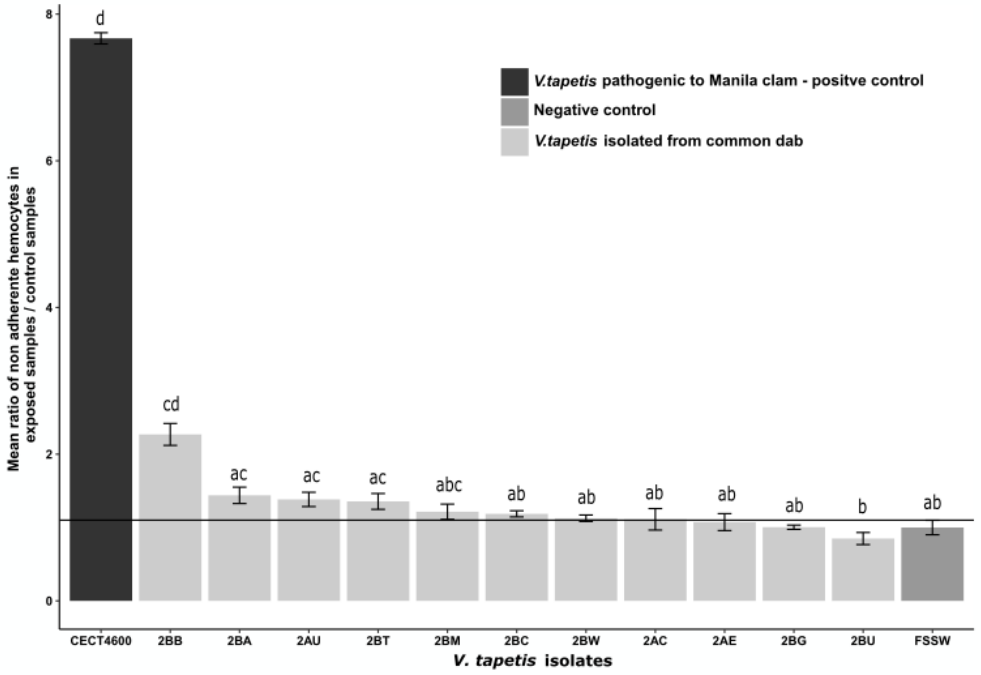

Figure 5.1: Effect of various isolates of V. tapetis on non-adherent cell ratio. Incubation time: 3h. Results are presented by a mean ratio of non-adherent hemocytes (i.e. round hemocytes) in presence of bacteria to the number of non-adherent cells after incubation with filtered sterile seawater (FSSW). Letters depict significant differences (Pairwise Student test). Errorbar = Standard Deviation/ √n (n = 3 replicates)

Comparison between different *in vitro* cytotoxicity bioassays is complicated since the non-adherent hemocyte ratio might vary depending on the susceptibility of hemolymph to *V. tapetis*. This is illustrated with the strain LP2, which showed intermediate cytotoxicity in Choquet *et al.* (2003) and negative cytotoxicity in Dias *et al.* (2018). In the present study, this bias risk was reduced, by using one pool of hemocytes and using a standardized analysis of the data comparing the results to both a positive (CECT4600) and negative control (FSSW), as already described in Choquet *et al.* (2003).

5.4.2 First detection of the *virB4* gene in non-cytotoxic fish isolates

Recently, a rapid and accurate Taqman real-time PCR assay for detection and quantification of *V. tapetis* in extrapallial fluids of the Manila clam has been developed, based on the presence of the *virB4* gene, which encodes a component of the T4SS (Bidault *et al.*, 2015). Since the T4SS has been described to be essential for virulence in other pathogenic species such as *Helicobacter pylori* and *Legionella pneumophila* (Voth *et al.*, 2012), this PCR analysis was suggested as a possible screening tool to differentiate between clam pathogenic and non-pathogenic isolates (Dias *et al.*, 2018). Among 17 fully sequenced *V. tapetis* genomes, in a previous study, the T4SS genes cluster was identified only in the genomes of isolates virulent to the Manila clam (based on *in vivo* assays), thus proving that these strains do not only carry the *virB4* gene but the entire T4SS gene cluster and also suggesting a role for this T4SS system in *V. tapetis* pathogenicity towards clams (Dias *et al.*, 2018). It should be noted that FPC1121, an isolate from Manila clam in Japan, does show *in vivo* virulence towards Manila clam, even though the *virB4* gene is not present. Surprisingly, in the isolates from common dab, the *virB4* gene was found to be present in seven out of the 11 isolates (2BM, 2AU, 2BA, 2BT, 2BU, 2AC and 2BG). This study is therefore the first to provide evidence that *V. tapetis* isolated from fish can carry the *virB4* gene, in contrast with previously reported results (Dias *et al.*, 2018). It needs however to be elucidated if these *virB4* positive isolates carry the entire cluster coding for T4SS and/or are able to induce BRD during *in vivo* experiments.

5.4.3 MALDI-TOF MS analysis reveals 3 clusters of *V. tapetis* isolates

All 27 *V. tapetis* isolates included in this study were analyzed using the MALDI-TOF MS method. This method allows sensitive and rapid identification of microorganisms and is now widely used in different fields such as clinical microbiology, epidemiological studies and water or food borne pathogens (Singhal *et al.*, 2015). The technique is broadly used to identify microorganisms at the species level, but has recently been shown valuable for strain typing (Sandrin *et al.*, 2013).

The main peaks generated by the MALDI-TOF MS were found between 2000 and 7500 Da. Based on the peak profiles, the constructed dendrogram (Figure 5.2) of the investigated spectra revealed some clearly delineated clusters. In total three distinct clusters (named A, B and C) were defined, based on differences in protein profiles (Figure 5.2). All strains that were derived from BRD outbreaks in cultured Manila clam between 1988 and 1996 clustered together in cluster B with only a 29.6 % similarity with other isolates. The strains derived from the common cockle (IS9) and from the carpet shell clam (RD0705) were also included in this cluster. All these strains were collected during the BRD emergence in Europe, which might explain the limited variability between the isolates. The two strains that were isolated from the rayed artemis (GDE) and pink clam (GTR-I) were clustered together with isolates from fish, in cluster A. The latter cluster could be further divided in four sub-clusters (Figure 5.2). Interestingly, one isolate (2BW) demonstrated different protein profiles and was included in a separate cluster C. This bacterium was isolated as a co-culture with *Pseudoalteromonas* sp. and *Psychrobacer submarinus* from a skin ulcer in dab. *In vitro* assays demonstrated the absence of the *virB4* gene and hemocyte cytotoxicity. Therefore, it is possible that this isolate represents a non-pathogenic strain for clams and/or dab. The virulence of this isolate should be characterized towards clams and fish to elucidate this clustering.

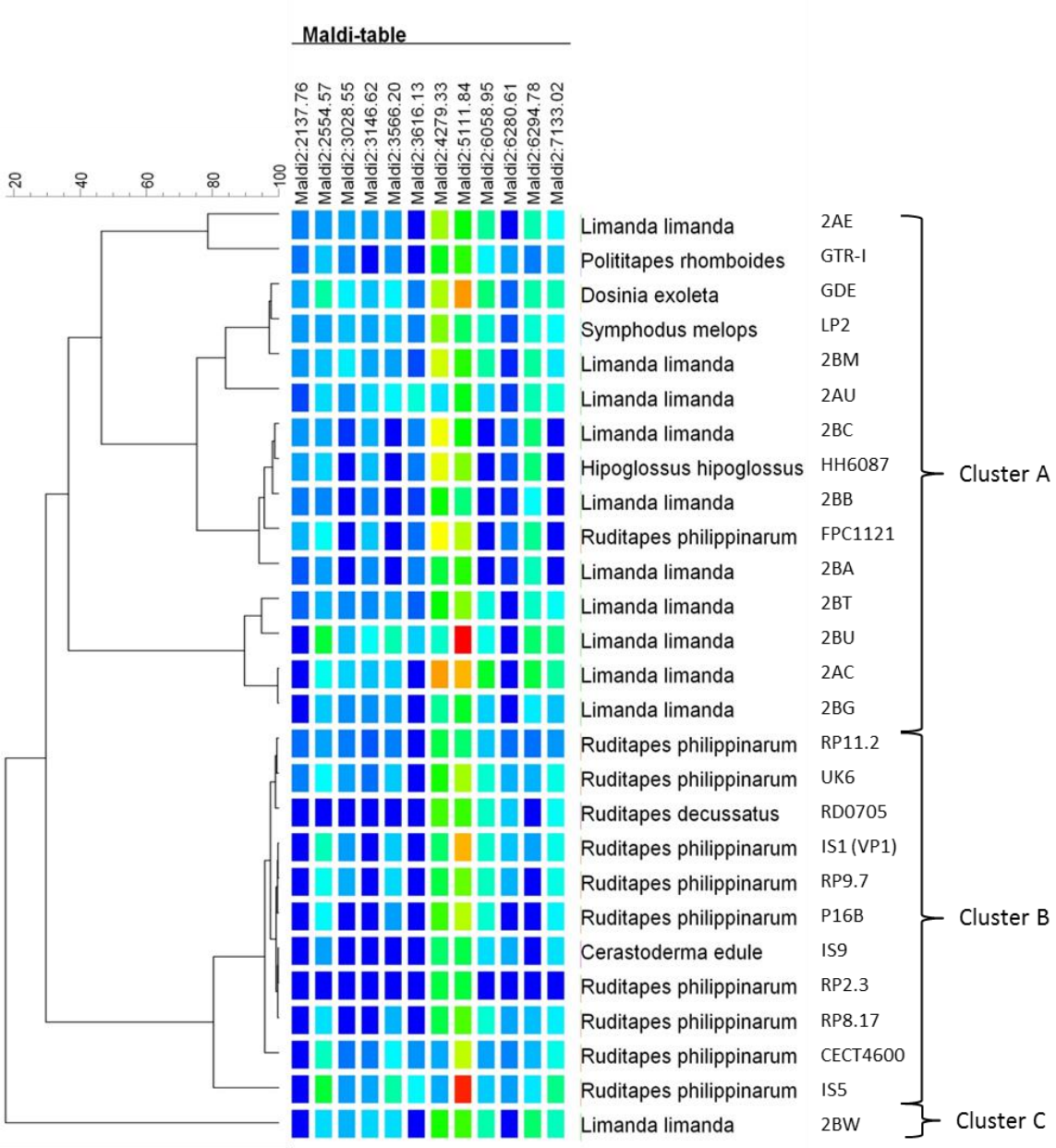

Figure 5.2: Dendrogram, based on the MALDI-TOF peak lists, of all V. tapetis isolates based on the complete spectra. Peak intensity is represented in the heat map using different colors ranging from blue (low intensity) to red (high intensity).

The MALDI-TOF MS clustering in the present study showed some similarities with previously reported data based on genome analyses (Dias *et al.*, 2018). The latter have demonstrated that the two strains isolated from the rayed artemis (GDE) and pink clam (GTR-I) clustered together with LP2 isolated from corkwing wrasse, and were genetically distant from *V. tapetis* strains isolated from clams.

FPC1121 is the only clam pathogenic strain that was clustered together with fish isolates, based on the MALDI-TOF MS profiling. FPC1121 was isolated from cultivated Manila clam in Japan (Table 5.1, Matsuyama *et al.*, 2010). The original report clearly described the brown deposit and mass mortalities of Manila clam due to this strain, which was confirmed by *in vivo* virulence assays (Matsuyama *et al.*, 2010). This clustering of FPC1121 is an interesting result; detailed genetic analysis would be interesting to explore the phylogenetic position of this strain and the linked genetic differences.

5.4.4 Value of the assays to discriminate isolates according to their host-species (Figure 5.3)

In vivo virulence tests are necessary to determine the ability for an isolate to induce BRD. However, several tests have been developed to characterize the *in vitro* virulence regarding Manila clam.

The *in vitro* cytotoxicity assay has been used as a tool to evaluate the pathogenicity of *V. tapetis* strains for clams (Bidault *et al.*, 2015; Dias *et al.*, 2018). This assay is based on the correlation between the cytotoxic activity of bacteria to clam hemocytes and the *in vivo* pathogenicity. As demonstrated in Table 5.1, such a correlation was not found for strain RD0705. Indeed, this strain causes no hemocytes cytotoxicity *in vitro* although it can cause BRD *in vivo* in the Manila clam (Table 5.1) (Novoa *et al.*, 1998; Dias *et al.*, 2018). Since RD0705 was isolated from another clam species (i.e. the grooved carpet shell), it is tempting to speculate that this inconsistency might be related to host specificity. However, it could also suggest that cytotoxicity towards hemocytes is not the only virulence factor involved in the development of BRD. Beside this exception, it should be recalled that the analysis of the virulence profiles of many *V. tapetis* strains isolated from clam revealed a good correlation between the cytotoxic activity to clam hemocytes and the *in vivo* pathogenicity.

Another assay commonly used to detect *V. tapetis* isolates pathogenic to clams is the search for the *virB4* gene encoding part of the T4SS (Dias *et al.*, 2018). In our study, we have demonstrated for the first time that the *virB4* gene can also be present in fish isolates. Since these isolates did not display *in vitro* virulence to clam hemocytes and they clustered separately based on their protein profile, it could be possible that they are not pathogenic for clams. *In vivo* studies should be performed to confirm the pathogenicity towards clams and if so, this might question the use of *virB4* detection as a pertinent marker for pathogenicity to clams.

The MALDI-TOF dendrogram showed a good clustering of *V. tapetis* from different origins. However, this clustering was not correlated with the presence of the *virB4* gene. In fact, the clustering was more correlated with the host species from which they were isolated. Based on the results of the different assays in the present study, it could be hypothesized that MALDI-TOF clustering could differentiate between clam pathogenic and non-pathogenic isolates.

5.4.5 Implications in the context of virulent strain detection

Based on these results, MALDI-TOF MS analysis seems to be a promising tool to indirectly evaluate the pathogenicity of a *V. tapetis* isolate towards the Manila clam. Nevertheless, although the presented MALDI-TOF MS isolate typing might be a rapid, cost-effective and powerful tool in identifying *V. tapetis* isolates, the Taqman real-time qPCR remains necessary to quantify the load of *V. tapetis* during infection. Although some inconsistencies exist, the *in vitro* assay is also believed to give a good indication of virulence towards Manila clam in some cases. Regarding the value of each test described in this study to determine *V. tapetis* isolates pathogenicity to the Manila clam, we can consider the MALDI-TOF MS as a new promising screening tool which can be complementary to the tools already used, increasing reliability of the screening.

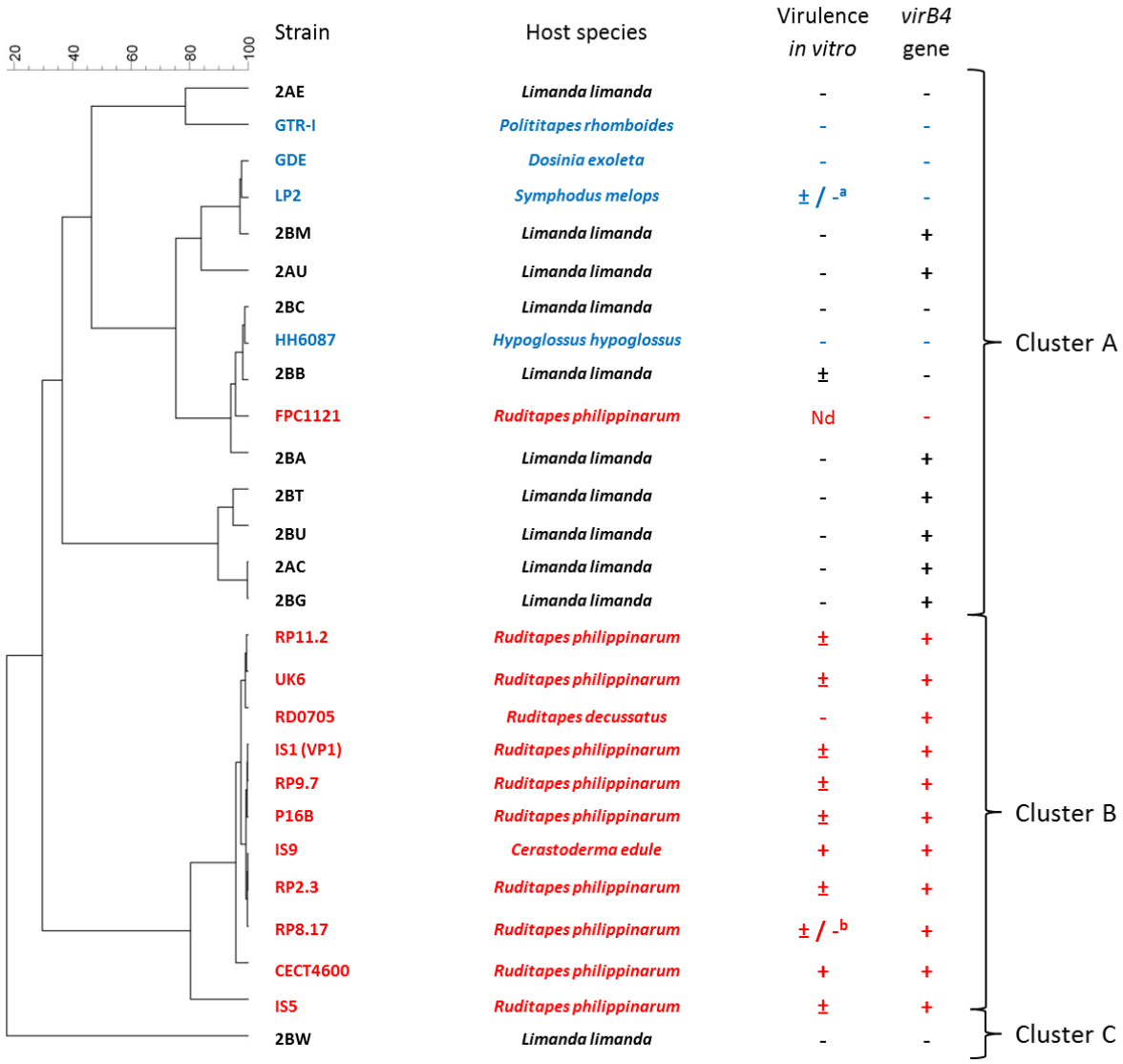

Figure 5.3: Dendrogram, based on the MALDI-TOF peak lists, of V. tapetis isolates derived from bivalves and fish combined with in vitro virulence (hemocyte cytotoxicity assay) and presence of the virB4 gene. In red: isolates that can induce Brown Ring Disease in vivo after injection in the pallial cavity. In blue: isolates that cannot induce Brown Ring Disease after injection in the pallial cavity. In black: isolates where no in vivo assay has been performed against the Manila clam. References of the virulence profiles can be found in Table 5.1. Cytotoxicity in vitro is depicted as follows: (-): no cytotoxicity, (±) intermediate cytotoxicity, (+): comparable cytotoxicity as the reference strain (CECT4600). a: negative in Dias et al. (2018) but intermediate in Choquet et al. (2003); b: negative in Dias et al. (2018) but intermediate in Choquet et al. (2004). Presence (+) or absence (-) of the virB4 gene as assessed using the TaqMan PCR method is also included. Nd: Not determined

In conclusion, the discovery of new *V. tapetis* isolates derived from common dab was linked with the need for a further exploration of variability of *V. tapetis* isolates. The currently used assays (toxicity to hemocytes and search of the *virB4* gene), have previously been shown to be interesting to discriminate between *V. tapetis* pathogenic and non-pathogenic clam isolates. Nevertheless, in this study, *V. tapetis* isolates from dab showed inconsistencies with results of previously pinpointed techniques, hereby questioning their discriminative power. In contrast, the MALDI-TOF MS analysis proved to be a promising

tool with the possible ability to differentiate between pathogenic and non-pathogenic clam isolates. This can be used as a complementary discriminative test for virulence of clam isolates. This approach allows rapid and cost-efficient identification of *V. tapetis* species and opens new perspectives to study the virulence of *V. tapetis* isolates but also to perform environmental monitoring in order to prevent outbreaks.

5.5 Acknowledgements

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Table 5.1: Information on the V. tapetis isolates of clams, other bivalves and fish with host species, common name of the host species, description of the health of the host species, year and location of isolation and a reference for each isolate. Furthermore, a synthesis is provided of the existing knowledge on (1) in vivo virulence, estimating the possibility of the isolate to cause Brown Ring Disease in Manila clam following experimental infection. The isolate indicated with an asterisk is able to cause Brown Ring Disease in pink clam but not in Manila clam; (2) in vitro cytotoxicity against hemocytes of the Manila clam ((-): no cytotoxicity, (±) intermediate cytotoxicity, (+):cytotoxicity. a: negative in Dias et al. (2018) and intermediate in Choquet (2004). b: negative in Dias et al. (2018) and intermediate in Choquet et al. (2003) And (3) presence of the virB4 gene from Bidault et al. (2015); (+) : presence, (-): absence. References for the virulence analyses are: Novoa et al. (1998); Choquet et al. (2004); Matsuyama et al. (2010); Bidault et al. (2015); Dias et al. (2018). Nd: Not determined; TS: Determined in this study.

Isolates	Host species	Common name	Isolated from	Year	Location	Isolate reference	*In vivo* virulence (1)	*In vitro* cytotoxicity (2)	*virB4* gene detection (3)
CLAMS									
RD0705	*Ruditapes decussatus*	Grooved carpet shell	Animals with BRD signs	1992	Spain, Galice	(Novoa et al., 1998)	+	–	+
CECT4600	*Ruditapes philippinarum*	Manila clam	Animals with BRD signs	1990	France, Landeda	(Borrego et al., 1996; Paillard and Maes, 1995)	+	+	+
FPC1121	*Ruditapes philippinarum*	Manila clam	Animals with BRD signs	2008	Japan	(Matsuyama et al., 2010)	+	Nd	–
IS1 (VP1)	*Ruditapes philippinarum*	Manila clam	Animals with BRD signs	1988	France, Landeda	(Paillard and Maes, 1990)	+	±	+
IS5	*Ruditapes philippinarum*	Manila clam	Animals with BRD signs	1991	France, Landeda	(Borrego et al., 1996)	+	±	+
P16B	*Ruditapes philippinarum*	Manila clam	Animals with BRD signs	1995	France, Golfe du Morbihan	(Allam et al., 2002)	+	±	+
RP11.2	*Ruditapes philippinarum*	Manila clam	Animals with BRD signs	1990	France, Landeda	(Borrego et al., 1996)	+	±	+
RP2.3.	*Ruditapes philippinarum*	Manila clam	Animals with BRD signs	1990	France, Landeda	(Borrego et al., 1996)	+	±	+
RP8.17	*Ruditapes philippinarum*	Manila clam	Animals with BRD signs	1990	France, Landeda	(Borrego et al., 1996)	+	±/–[α]	+
RP9.7	*Ruditapes philippinarum*	Manila clam	Animals with BRD signs	1990	France, Landeda	(Borrego et al., 1996)	+	±	+
UK6	*Ruditapes philippinarum*	Manila clam	Animals with BRD signs	1996	Great Britain	(Allam et al., 2000)	+	±	+
OTHER BIVALVES									
IS9	*Cerastoderma edule*	Common cockle	Healthy animals	1990	France, Quiberon	(Borrego et al., 1996)	+	+	+
GTR-I	*Polititapes rhomboïdes*	Pink clam	Animals with BRD signs	2008	France, Glénan	(Dias et al., 2018)	–*	–	–
GDE	*Dosinia exoleta*	Rayed artemis	Animals with BRD signs	2003	France, Glénan	(Dias et al., 2018)	–	–	–
FISH									
LP2	*Symphodus melops*	Corkwing wrasse	Kidney of fish suffering vibriosis	1999	Norway, Bergen	(Jensen et al., 2003)	–	±/–[β]	–
HH6087 (CECT8161)	*Hipoglossus hipoglossus*	Atlantic halibut	Kidney of moribund fish	2002	Great Britain, Glasgow	(Reid et al., 2003)	–	–	–
2AC	*Limanda limanda*	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	TS	TS
2AE	*Limanda limanda*	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	TS	TS
2AU	*Limanda limanda*	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	TS	TS
2BA	*Limanda limanda*	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	TS	TS
2BB	*Limanda limanda*	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	TS	TS
2BC	*Limanda limanda*	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	TS	TS
2BG	*Limanda limanda*	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	TS	TS
2BM	*Limanda limanda*	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	TS	TS
2BT	*Limanda limanda*	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	TS	TS
2BU	*Limanda limanda*	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	TS	TS
2BW	*Limanda limanda*	Common dab	Skin ulceration	2017	Belgium (North sea)	(Vercauteren et al., 2018)	Nd	TS	TS

Chapter 6

Role of *Aeromonas salmonicida* in skin ulceration development

Based on: Vercauteren, M**.,** De Swaef, E., Declercq, A. M., Aerts, J., Ampe, B., Gulla, S., Haesebrouck, F., Devriese, L., Decostere, A., and Chiers K. (2019). Pinpointing the role of Aeromonas salmonicida in the development of skin ulcerations in common dab (*Limanda limanda)*. *Journal of Fish Diseases*. 43, 347-357.

6.1 Abstract

Aeromonas salmonicida was isolated from ulcerations in common dab (*Limanda limanda*). An experiment was performed to pinpoint its role in ulceration development, considering the importance of the skin barrier and the pigmented and non-pigmented side. The skin of dab was treated in three zones, one where scales and epidermis was removed, one where mucus was discarded and one non-treated zone. Fish were tagged to allow individual identification and challenged with *A. salmonicida*. Mortality and severity of the developing lesions were recorded for 21 days post-inoculation.

Starting 12 days post-inoculation, mortality occurred gradually in challenged fish, however, no direct cause could be established. Both control and challenged fish developed ulcerations containing *A. salmonicida. S*equencing of *vap*A gene revealed that isolates retrieved from both groups were distinct, suggesting the presence of *A. salmonicida* prior to the trial. Most ulcerations developed in zones where skin was removed, suggesting that abrasion might be a predisposing factor in ulceration development. Ulcerations were also observed at the insertion site of the tag, where exposed muscle tissue might have favored development of ulcerations. In conclusion, *A. salmonicida* seems to be involved in the development of skin ulcerations in dab, although the exact pathogenesis needs to be elucidated.

6.2 Introduction

Ulcerative skin lesions are commonly reported in many freshwater fish such as pike (*Esox Lucius*) (Wiklund, 1990), goldfish (*Carassius auratus*) (Elliott & Shotts, 1980), carp (*Cyprinus carpio*) (Fijan, 1972) and various salmonid species (Austin and Austin, 2012), but also in commercially important marine flatfish such as turbot (*Scophthalmus maximus*) (Toranzo & Barja, 1992; Pedersen *et al.*, 1994) and flounder (*Platichthys flesus*) (Vethaak, 1992; Wiklund & Bylund, 1993; Wiklund *et al.*, 1999). Prevalences up to 3.5 % were reported in flatfish populations in the Belgian part of the North Sea (BNS) (Devriese *et al.*, 2015). In the Northern North Sea, Baltic Sea and around Iceland, a prevalence ranging between 0.8 % and 7.2 % was noted (Lang *et al.*, 2017). Besides representing a welfare issue, these lesions might pose limitations to the economic productivity of the marine ecosystem (Bouck & Smith, 1979; Noga, 2000). The etiology of such skin ulcerations in flatfish species has not yet been fully elucidated. A complex and multifactorial etiology with possible involvement of infectious agents, biological toxins, aspects of the fish and environmental factors (Wiklund, 1994; Noga, 2000; Law, 2001), might be expected. Disruption of the mechanical, chemical and immunological barrier formed by the skin and mucus, caused by abrasion or pollution, may increase the susceptibility to bacterial infection and subsequent skin ulceration development. Indeed, it has been previously demonstrated that *Vibrio tapetis* can cause ulceration of damaged skin in common dab (*Limanda limanda*) with the pigmented side being seemingly predisposed (Vercauteren *et al.*, 2019a).

In some cases, skin ulcerations were linked to an infection with the Gram-negative bacterium *Aeromonas salmonicida*, described as one of the most harmful invasive bacteria by the Delivering Alien Invasive Species Inventory for Europe (DAISIE) project (Virsek *et al.*, 2017). *A. salmonicida* was presumably introduced in Sweden with the translocation of fish, the exact origin is unknown (Katsanevakis *et al*., 2012). This bacterial species is a well-studied fish pathogen causing systemic disease in various fresh and salt water fish species around the world (Austin and Austin, 2012). Commonly reported clinical signs linked to *A. salmonicida* infections include skin ulcerations, furuncle formation, lethargy, loss of orientation, hemorrhages in various internal organs, splenomegaly, myocarditis, liquefactive necrosis of the kidney and mortality (Wiklund & Dalsgaard, 1998; Austin & Austin, 2012). Recently, *A. salmonicida* was isolated from skin ulcerations in common dab in the BNS (Vercauteren *et al.*, 2018). Various subtypes of the pathogen have been described and may be discerned based on the sequence of the virulence array protein gene (*vapA*), producing the crystalline, outer membrane protein (A-layer) (Gulla *et al.*, 2016). The isolates recovered from common dab in the BNS could all be assigned to the distinct A-layer type 15 (Vercauteren *et al.*, 2018), a type which has subsequently been identified as exclusively associated with Northern European specimens of this fish species (Gulla *et al.*, 2019).

Due to the economic burden from losses caused by *A. salmonicida*, a vast amount of information exists on genomic diversity, taxonomy, virulence and host range of the pathogen. However, a strong historical focus has been on strains causing disease in salmonid fish, and the role of *A. salmonicida* in the development of skin ulcerations in common dab has not yet been elucidated.

In this framework, an experimental challenge using bath immersion was performed to pinpoint the role of *A. salmonicida* A-layer type 15 in the development of skin ulcerations in common dab taking

into account the possible role of the skin barrier. Additionally, a possible predisposition for the pigmented and/or non-pigmented side was assessed.

6.3 Materials and methods

6.3.1 Animals and housing

Sixty common dab were caught on board of the Research Vessel (RV) Simon Stevin using a 6 m beam trawl. Short fishing hauls (10 - 15 min) were used at two sampling locations (L1 (N51°10.344; E2°38.699); L2 (N51°9.886; E2°34.797)) in the BNS. Fish in good condition and with a minimal size of 17 cm were placed in a survival tank (1 x 1.2 x 1 m; 640 L) with a constant influx of fresh sea water. Within four hours after catch, fish were transported to the Marine Station of Ostend (MSO, Flanders Marine Institute (VLIZ)) for acclimatization where they were held in two circular aquaria (diameter 2.6 m, 4000 L) with recirculating, filtered natural sea water. On the bottom of the aquaria a sediment layer (± 6 cm layer thickness, 0-2 mm grain size) was foreseen. The quality of the seawater was monitored daily and kept in pre-set ranges (pH 8.0 ± 0.1; 71.2 ± 1.9 % oxygen saturation; and 29.2 ± 0.1 PSU). Ammonia and nitrite levels never exceeded 0.1 and 1 mg L^{-1}, respectively. Temperature of the water started at 8 ± 1 °C upon arrival of the fish and was increased up to 16 ± 1 °C in due course (+ 1°C per 48 hours). Fish were fed three times a week with chopped whiting (*Merlangius merlangus*) (10 g per fish). After 20 days, all fish were transported to the experimental units at the Faculty of Veterinary Medicine (Ghent University) using transportation boxes (39.4 x 59.8 x 18.6 cm; 30 L) filled with natural seawater and supplied with an oxygen tablet (JBL GmbH & Co.KG, Germany).

Upon arrival at the experimental units, all fish were randomly divided over five experimental tanks (1 x 1 x 0.5 m; 450 L; 12 fish per tank) with recirculating seawater (combination natural and artificial seawater). In each tank a sediment layer (± 6 cm layer thickness, 0-2 mm grain size) was provided. Water parameters were monitored daily and the water was replaced by aerated artificial sea water, when needed, to keep the water parameters within acceptable ranges (14.4 ± 0.3 °C; pH 8.4 ± 0.02; 84.3 ± 0.7 % oxygen saturation; and 30.9 ± 0.1 PSU). Fish were fed every two days with small pieces of Pangas catfish (*Pangasius pangasius*) (10 g per fish). Feeding time lasted for one hour after which the remaining feed was removed. For twenty-four hours prior to the experimental trial, fish were deprived of food. The experimental design was approved by Ethical Committee of the Faculty of Veterinary Medicine and Bio-engineering Sciences, Ghent University (EC 2015_89).

6.3.2 Bacterial strain

The bacterial strain (Original isolate – 2CK) was isolated from an active skin ulceration in a common dab caught in the BNS. The isolate was identified as *A. salmonicida* using 16S rRNA gene sequencing and subsequent *vap*A-gene sequencing confirmed the presence of A-layer type 15 (Vercauteren *et al.,* 2018). According to the traditional nomenclature, the isolate was an 'atypical' strain, however it did not belong to any of validly described subspecies. For the experimental challenge, the *A. salmonicida* isolate was cultured on Tryptic Soy Agar (TSA, Sigma Aldrich N.V., Belgium) incubated at 16 ± 1 °C. After three days, colonies were harvested and suspended in four mL Tryptic Soy Broth (TSB, Sigma Aldrich N.V., Belgium; 16 ± 1°C). Following 48 h incubation, the broth was used to inoculate 200 mL TSB medium (16 ± 1 °C) and centrifuged (2465 x *g*, two times 10 min, 16 °C) after another 48 h incubation. The pellet was re-suspended in 40 mL autoclaved artificial seawater. Bacterial titers were verified by making a tenfold dilution series in triplicate on TSA, prior to administration.

6.3.3 Experimental design

All fish were anesthetized using Tricane Methanesulfonate (MS-222, 100 mg mL^{-1} seawater, 124 ± 37 s) after which a tag (T-bar anchor tag, Floy Tag Inc., USA) was inserted in the caudal epaxial musculature. All fish were weighed (W_b) and the sex of the fish was determined. Additionally, lesions present before the start of the experiment were listed and photographed.

The skin of all fish was treated in three 'treatment zones' (each ± 2.3 cm^2, 0.5 cm interspace) on both pigmented (P) and non-pigmented (NP) sides (Vercauteren *et al.*, 2019a). One zone was mechanically treated (MT) whereby the scales and overlying epidermis were removed by scraping with a scalpel. In the second zone, the chemical treatment (CT), the mucus layer on top of the epidermis was removed using ethanol. The third zone served as a non-treatment zone (NT) where skin was left intact. The sequence of all treatments was altered on each fish, resulting in six subgroups (1-6) of 10 fish each; (subgroup 1) MT – CT – NT; (2) MT – NT – CT; (3) NT – MT – CT; (4) NT – CT – MT; (5) CT – NT – MT; (6) CT – MT – NT (Vercauteren *et al.*, 2019a).

Following recovery of the fish after anesthesia, fish were divided into two groups; one which was to be challenged with *A. salmonicida* (three tank replicates) and one sham treated control group (two tank replicates). Each tank replicate, henceforth referred to as replicate, contained 12 fish that were placed in separate tanks (55 x 38 x 28 cm, 35L) containing artificial seawater. The bacterial suspension of *A. salmonicida* was added to the water of the replicates of the challenge group leading to a final concentration of 3.7 x 10^6 colony forming units (CFU) mL^{-1} per tank. The two control group replicates were treated similarly without the addition of *A. salmonicida*. After one hour, the fish were transferred to the experimental tank (12 fish per tank). During a 21-day period following inoculation, mortality and clinical signs were recorded daily. Every two days, fish were netted, clinically inspected and photographed on both the P and NP sides to determine the "Gross Ulceration Score" (GUS), "Total Affected Area" (TA, cm^2), "Ulcerative Area" (UA, cm^2) and "Tag Ulceration Score" (TUS) (see further).

Moribund fish with extensive lesions, subcutaneous hemorrhages and no reaction to stimuli were euthanized (MS-222; 500 mg L^{-1} seawater) and necropsied as described below. All remaining fish were sacrificed at 21 days post-inoculation (DPI) using an overdose of MS - 222 (500 mg L^{-1}) and necropsied as described below.

6.3.4 Post mortem examination

Fish-related characteristics such as length (L), weight (W_E), and sex were recorded upon necropsy. The Fulton condition index (K_E= 100*(W_E/L^3); Fulton, 1904) was calculated at the end of the experiment, allowing comparison with the body condition of the fish before treatment (K_B= 100*(W_B/L^3)). Sagittal otoliths were collected to determine the age of the fish (Imsland *et al.*, 2014). Wet mount preparations of gill biopsies and skin mucus samples were examined to estimate their parasitic load. For each sample the number of parasites was counted in one microscopic field (x 40, High Power Field (HPF)).

Both on P and NP sides, treatment zones were photographed to determine the GUS, TA and UA (see further). Lesions outside the treatment zone and at the insertion site of the tag were photographed as well as to study the severity and to calculate the TUS, respectively.

A full necropsy of the fish was performed whereby samples of skin lesions and internal organs were collected for further bacteriological and histological examination.

6.3.5 Skin lesion assessment

Gross Ulceration Score (GUS)

The severity of the lesions developed in the treatment zones (MT - CT - NT) was analyzed using a previously described scoring system (Supplementary file 1; Vercauteren *et al.*, 2019a). This was based on seven parameters - depth, healing, elevation of the edge, pigmentation, color, bleeding in the edge, and the form of the lesion. The scores of all parameters were summed resulting in a GUS whereby a higher value indicates a more severe lesion. The scoring was conducted blindly, based on photographs taken every two days during the experimental period and at necropsy. Moreover, the severity of the lesion at the insertion site of the tag (TUS) was also scored (0 = no reaction observed - 3 = Large lesion (> 0.5 cm).

Total Affected Area (TA) and Ulcerative Area (UA)

To quantify the extent of the ulceration in the treatment zones (MT – CT – NT), the area of the fish's skin affected by each lesion was determined as previously described by Vercauteren *et al.* (2019a). The TA (cm^2), representing the total skin area affected by the ulceration, including the healing edge and/or hemorrhages around the lesion, as well as the UA (cm^2), being the surface of the open, active lesion, was calculated for each zone using scientific image analysis software (ImageJ 1.4).

6.3.6 Bacteriological examination

Samples from ulcerations within the treatment zones (MT – CT – NT), internal organs and lesions outside the treatment zones were inoculated on TSA (Sigma Aldrich N.V., Belgium) using an inoculation loop and incubated for three days at 16 ± 1 °C. *A. salmonicida* was identified on purified cultures using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) method. A-layer typing based on sequence heterogeneity in the *vapA* virulence gene was performed on 21 (out of 61) *A. salmonicida* isolates (Gulla *et al.*, 2016) from both control and challenged fish that were sacrificed at 21 DPI. DNA extraction, PCR (primers *vapA* F2 and R3), Sanger sequencing and subsequent processing were performed as previously described by Gulla *et al.* (2016, 2019). The partial *vapA* sequence from the challenge strain 2CK (accession no. MK244113), and from isolates 18 (accession no. MK244206) and NVI-09978 (accession no. MK243982), respectively recovered from common dab in Denmark in 1999 and Atlantic halibut (*Hippoglossus hippoglossus*) in Norway in 2015 (Gulla *et al.*, 2019), were acquired from NCBI GenBank and included for comparison and tree rooting. Sequence alignments were conducted in ClustalX v2.1 (Larkin *et al.*, 2007) and a maximum likelihood (ML) tree was constructed using PhyML v3.0 (Guindon *et al.*2010) with default settings. The ML tree was edited in FigTree v1.4.3 (tree.bio.ed.ac.uk/software/figtree).

6.3.7 Histological examination

Skin samples of ulcerations in the treatment zones (MT – CT – NT), as well as samples of gill, liver, spleen, intestine, kidney and heart, were fixed for 24 h in a phosphate-buffered 4 % formaldehyde solution. Tissues were processed according to standard techniques, sectioned (5 µm) and stained with hematoxylin and eosin (H & E). Histological examination of the ulcerations was performed to confirm the gross appearance of the lesions characterized according to Vercauteren *et al.* (2019a).

6.3.8 Data processing and statistical analysis

To investigate the difference in fish-related characteristics between control and challenged fish, length, weight, body condition and age were used as response variables in a linear mixed model (proc GLIMMIX). The parasitic load in the gills was analyzed using a linear mixed model following a

Poisson distribution. Due to the lack of parasites on the skin of the control fish, different statistical method was applied. The probability to have a 0 % prevalence of skin parasites was calculated using the probability mass function with binomial distribution. Differences in mortality between both groups were studied using logistical regression. Tank replicate was included as a random intercept in all models.

To study the role of *A. salmonicida* in the development of skin ulcerations, statistical analyses were performed in fish that survived until the end of the experimental period (21 DPI). Results of fish that died prior to the end of the experiment were reported in a descriptive manner. For estimating the difference in GUS, TA, UA and TUS, a linear mixed model (proc GLIMMIX) was used, followed by a pairwise comparison using a Tuckey-Kramer adjustment for multiple testing. GUS, TA, UA and TUS were used as response variables. Different treatments (MT – CT – NT), groups, replicates, and subgroups (variable order of the treatments) and side (P - NP) were implemented as variables. All analyses were stratified by day. Analysis on differences between P and NP side was performed in the same manner with side and group as response variables. Interaction effects were studied to estimate group-dependency. In all models, tank replicate was included as a random intercept. These statistical analyses were performed using SAS 9.4.

Table 6.1: Main fish characteristics (length, weight, body condition, sex and age) per tank replicate and per group, as well as the mortality in each replicate during the experimental period.

Group	Replicate	No.	Length (cm)	Weight (g)		Body condition		Sex	Age (months)	Mortality	
				W_B	W_E	K_B	K_E			< 10 DPI	< 21 DPI
Challenge	1	12	20.3 ± 2.4	87.1 ± 33.4	83.3 ± 33.8	1.0 ± 0.1	1.0 ± 0.1	2 M / 10 F	45.4 ± 13.9	0	3
	2	12	19.8 ± 1.7	81.7 ± 17.4	72.1 ± 13.4	1.0 ± 0.1	0.9 ± 0.1	1 M / 11 F	41.0 ± 6.0	0	3
	3	12	21.8 ± 2.8	110.4 ± 49.3	104.5 ± 45.8	1.0 ± 0.1	1.0 ± 0.1	1 M / 11 F	50.0 ± 14.0	0	4
	Mean		**20.6 ± 2.5**	**93.1 ± 37.0**	**87.1 ± 35.9**	**1.0 ± 0.1**	**1.0 ± 0.1**	**4 M / 32 F**	**45.9 ± 12.4**		
Control	1	12	23.0 ± 2.5	123.2 ± 46.1	116.5 ± 43.5	1.0 ± 0.1	0.9 ± 0.1	0 M / 12 F	46.8 ± 11.8	0	1
	2	12	22.1 ± 2.9	110.2 ± 42.8	106.6 ± 41.8	1.0 ± 0.1	0.9 ± 0.1	1 M / 11 F	46.0 ± 10.4	0	0
	Mean		**22.6 ± 2.7**	**116.7 ± 44.0**	**111.5 ± 42.0**	**1.0 ± 0.1**	**1.0 ± 0.1**	**1 M / 23 F**	**46.4 ± 10.8**		

W_B: Weight at the beginning of the experimental period; W_E: Weight at the end of the experimental period; K_B: Fulton condition index at the beginning of the experimental period; K_E: Fulton condition index at the end of the experimental period; M: male fish; F: female fish.

Differences were considered to be significant when p-values were lower than 0.05. A p-value between 0.05 and 0.1 was considered a trend. The analyzed continues data were considered to be sufficiently normally distributed, based on the graphical evaluation (histogram and QQ-plot) of the

residuals. When the data was not sufficiently normally distributed a non-parametric alternative was used. All statistical analyses were performed using SAS 9.4.

6.4 Results

Table 6.1 lists an overview of the main fish characteristics. The mean length of the fish was 20.6 ± 2.5 cm (4 males, 32 females) and 22.6 ± 2.7 cm (1 male, 23 females) in the challenge and control group, respectively. The mean age of the fish in the challenge (45.9 ± 12.4 months) was not significantly different from the control group (46.4 ± 10.8 months) (p = 0.8894). At the beginning of the experiment, the mean body condition of the challenge (K_B = 1.0 ± 0.12) and control group (K_B = 0.97 ± 0.08) was not different (p = 0.3470) and the body condition of both groups remained stable during the experiment (K_B - K_E) (p = 0.4927).

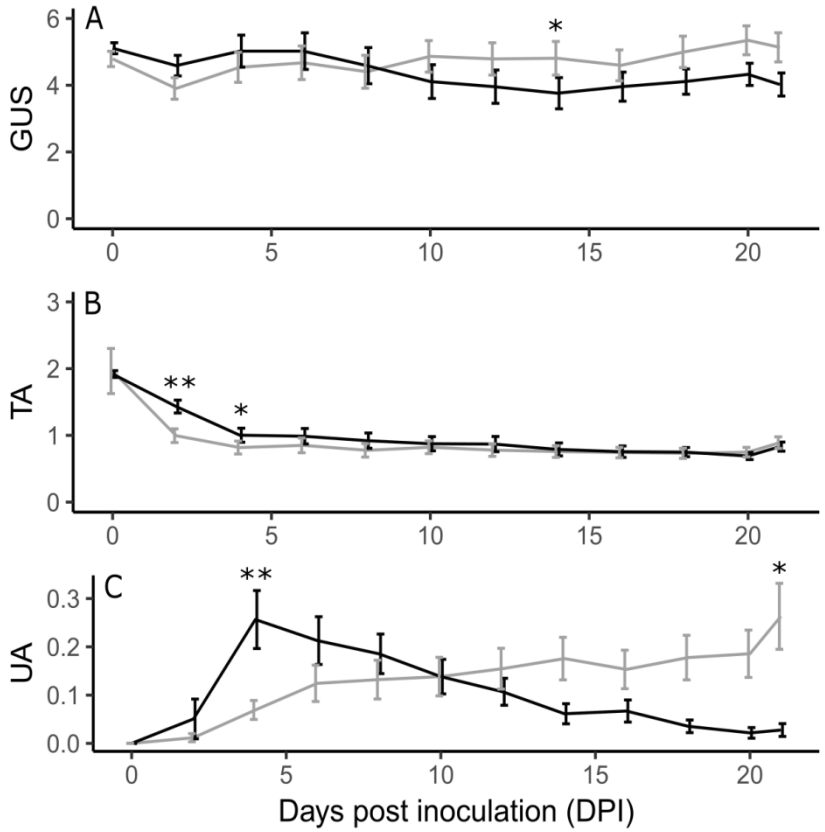


Figure 6.1: Overview of skin lesion assessment for mechanically treated zones during the experimental period. Comparison (mean ± se) of the (A) Gross ulceration score (GUS), (B) Total affected area (TA, cm²) and (C) Ulcerative area (UA, cm²) between challenge (grey) and control (black) groups over the total experimental period (Days post-inoculation, DPI). Significant differences are indicated with two asterisks (**) and a trend is indicated with one asterisk (*). Only surviving fish were evaluated.

Skin parasites, mainly identified as *Trichodina* spp., were present in the mucus of five challenged fish and none of the control fish. The prevalence of skin parasites in the control group is smaller compared to the challenge group (p = 0.0013). Twenty-one out of 48 studied gill biopsies contained parasites morphologically similar to *Tetrahymena* spp*., Costia* spp. or *Trichodina* spp.. The parasitic load in the gills (light to moderate; 1 - 8 parasites per HPF) was higher in challenge (2.77 ± 1.07 parasites/ HPF) compared to control group (0.86 ± 1.46 parasites /HPF) (p = 0.0007).

Length (p = 0.1030), weight (W_B: p = 0.2989; W_E: p = 0.1474) and body condition and were not considered in further statistical analyses since no significant differences between challenged and control fish were observed. Sex was neither implemented in additional examinations due to a low amount of male fish. Some fish showed pre-existing lesions including small bulging lesions (four in challenge, one in control group), papilloma-like lesions (five in challenge group), skeletal deformities (two in challenge group), skin lesions (two in the control group) and/or lesions at the lower jaw (two in the control group), before the start of the trial. These pre-existing lesions were not implemented in the analyses.

Mortality in the challenge group (n = 9; 25.0 %) was significantly higher compared to the control group (n = 1; 4.2 %) (p = 0.0499). Mortalities started to occur at 12 DPI and lasted until 20 DPI. In the challenge group, one fish was euthanized due to extensive subcutaneous hemorrhages. In total 10 of 36 challenged fish died.

The microsporidian *Glugea* sp. was encountered in the intestines of both challenged (n = 2) and control fish (n = 3) (p = 0.6602). Except for this parasitic infection, necropsy revealed no macroscopic abnormalities neither in the internal organs nor gills in fish from either group.

6.4.1 Chronological changes in skin lesions at the different treatment zones

Non-treated (NT)-zone

No ulcerations developed in the NT-zones, neither in challenged or in control fish. At 21 DPI, *A. salmonicida* was isolated from the macroscopically intact NT-zone of one control and one challenged fish.

Chemical-treatment (CT)-zone

Two control and one challenged fish developed an ulceration during the experiment in the CT-zone. All ulcerations showed clear signs of healing at 21 DPI. *A. salmonicida* was not isolated from these ulcerations.

Mechanical-treatment (MT)-zone

0-10 days post-inoculation (DPI)

All control and challenged fish developed an ulceration in the MT-zone on one or both sides with a comparable GUS of 4.73 ± 0.38 in the control fish and 4.53 ± 0.34 in the challenged fish (Figure 6.1; Table 6.2 for detailed values). The control fish showed significantly higher TA on 2 DPI (p = 0.0457) compared to the challenged fish. On 4 DPI, a similar trend was present (p = 0.0947) which disappeared later on (Figure 6.1). On this day, the UA was also higher in the control fish compared to the challenged fish (p = 0.0403). However, this difference decreased later on.

The chronological changes related to the P and NP sides are visualized in Figure 6.2. Starting (0 DPI) with a similar GUS score on P (5.31 ± 0.71) and NP (4.57 ± 1.83), the lesions evolved with a steep increase in GUS between 2 DPI (5.57 ± 1.12) and 10 DPI (7.29 ± 1.47) on the P side. On the NP side, the opposite evolution was observed with a decrease in GUS over time (2 DPI: 2.88 ± 2.26; 10 DPI: 1.73 ± 2.40). A moderate decrease in TA was observed on the P side, while on the NP side, the decrease followed a steeper course. The UA showed a steep increase on the P side between 2 DPI

and 6 DPI with UA of 0.02 ± 0.09 cm² and 0.30 ± 0.37 cm², respectively. Between 6 and 10 DPI, the UA declined. On the NP side, the UA remained rather stable over time. Generally, ulcerations on the P side were more severe (GUS) and larger (TA, UA) compared to ulcerations on the NP side (Table 6.2).

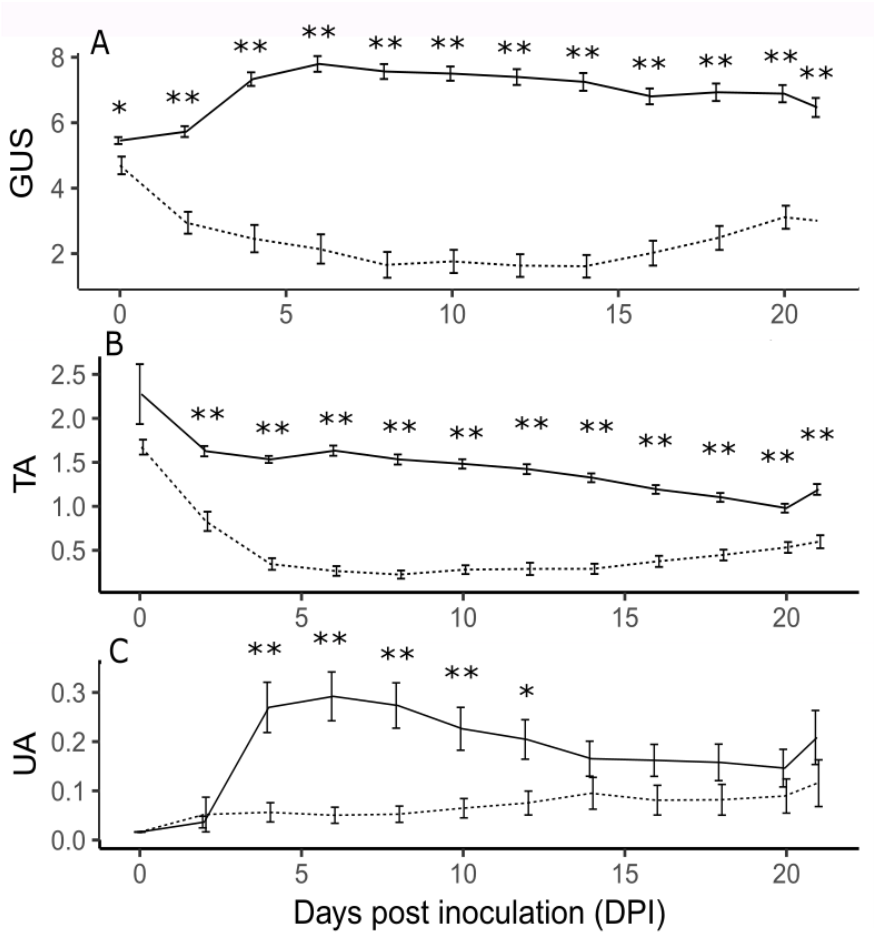


Figure 6.2: Overview of skin lesion assessment for mechanically treated zone ulcerations on pigmented and non-pigmented sides. Comparison (mean ± se) of the (A) Gross ulceration score (GUS), (B) Total affected area (TA, cm²) and (C) Ulcerative area (UA, cm²) between pigmented (full line) and non-pigmented (dotted line) side over the total experimental period (Days post-inoculation, DPI). Statistically significant differences are indicated with two asterisks (**) and a trend is indicated with one asterisk (*). Only surviving fish were evaluated.

11-21 days post-inoculation (DPI)

In the control group, one fish died at 14 DPI. This fish displayed one ulceration on the pigmented MT-zone. The ulceration had a GUS of 7 at necropsy and TA of 2.36 cm² whereby 0.92 cm² was active (UA). The fish developed three additional ulcerations outside the treatment zones and a lesion in the head region. *A. salmonicida* was not isolated from any of the lesions.

The fish of the challenge group that died (n = 10) all developed ulcerations in the MT-zone with a GUS of 6.16 ± 1.08, TA of 1.45 ± 0.09 cm² and UA of 0.57 ± 0.44 cm². One of these fish had a very large and severe ulceration in the MT-zone on the NP side (GUS: 15, TA: 28.93 cm²; UA: 13.05 cm²). *A. salmonicida* was not isolated from this ulceration. Two fish had one or more large ulcerations outside the treatment zones, which aggravated from 11 DPI onwards. *A. salmonicida* was not isolated from these lesions. In the other fish that died, *A. salmonicida* was isolated from only one ulceration in the MT-zone. Upon histological examination of the heart, signs of myocarditis were

found in three challenged fish. During the entire experimental period, there was no difference in GUS ($p = 0.5147$) and TA ($p = 0.3589$) between fish that died during the experiment and fish that survived. Fish that died showed a trend for a higher UA ($p = 0.0673$).

All remaining control fish (n = 23) exhibited ulcerations with a GUS of 4.02 ± 0.19, TA of 0.78 ± 0.06 cm² of which 0.05 ± 0.03 cm² was active (UA). Between 11 and 21 DPI, GUS remained stable until 20 DPI, where after a small decline was seen (Figure 6.1). TA remained stable and UA showed a vast decrease during this period (Figure 6.1). *A. salmonicida* was isolated from the ulcerations of four fish, yielding pure primary cultures in three cases.

The surviving challenged fish (n = 26) developed ulcerations with a GUS of 4.95 ± 0.27. The TA was 0.78 ± 0.06 cm² and UA was 0.19 ± 0.04 cm². The GUS and UA of the surviving challenged fish showed a small increase towards 21 DPI. At 14 DPI, the challenged fish showed a trend for a higher GUS ($p = 0.0864$) and UA ($p = 0.0993$) compared to the control fish (Table 6.2). *A. salmonicida* was isolated from the ulcerations of ten fish, resulting in pure cultures in five cases. For the remaining five animals, cultures harboring two or three colony types, including *A. salmonicida*, were obtained.

In surviving challenged fish, the GUS on the P side between 11 and 21 DPI remained stable with a small decrease after 14 DPI and towards the end of the experiment (Figure 6.2). On the NP side, the opposite reaction was observed with an increase between 14 DPI (1.59 ± 2.34) and 21 DPI (2.94 ± 2.59) (Table 6.2). For the TA, the same trend as before was observed, with a small decline in TA on the P side and a slight increase on the NP side (Figure 6.2). Starting at 14 DPI, the UA was comparable between both sides (14 DPI: $p = 0.2425$; 18 DPI: $p = 0.2128$).

Ulcerations were histologically identified by focal loss of epidermal and/or dermal tissue. Infiltration of inflammatory cells was found in the dermal tissue. In most cases, the underlying muscular tissue showed signs of degeneration and infiltration of inflammatory cells. In a minority of cases (n = 11), the infection remained more superficial affecting only dermal tissue. Partially healed ulcerations were mainly recognized by a one or two-layered epidermal tissue covering the degenerated dermal tissue. These observations were similar in the challenge and control group, and on both sides of the fish.

6.4.2 Chronological changes in skin lesions at the insertion site of the tag

0-10 days post-inoculation (DPI)

In 11 fish of replicate two of the control group, skin lesions were not observed at the insertion site of the tag. The remaining fish of this replicate showed a small reddening of the skin at this site, which remained stable throughout the entire experiment. In all fish of the other replicates (n = 48), lesions at the insertion site of the tag evolved from small hemorrhages (at 2 DPI) to small lesions (at 10 DPI; Figure 6.3); TUS of 0.82 ± 0.39 (control group – replicate one) and 0.78 ± 0.05 (challenge group) at 2 DPI, and 1.64 ± 0.90 (control group – replicate one) and 1.72 ± 0.13 (challenge group) at 8 DPI (Figure 6.4). No statistical difference between challenge and control group was observed between 2 and 8 DPI. At 10 DPI, the TUS of the challenged fish showed a steeper incline in TUS compared to the fish of the control group, resulting in a trend indicating a higher TUS in the challenged fish compared to the control fish (Figure 6.4).

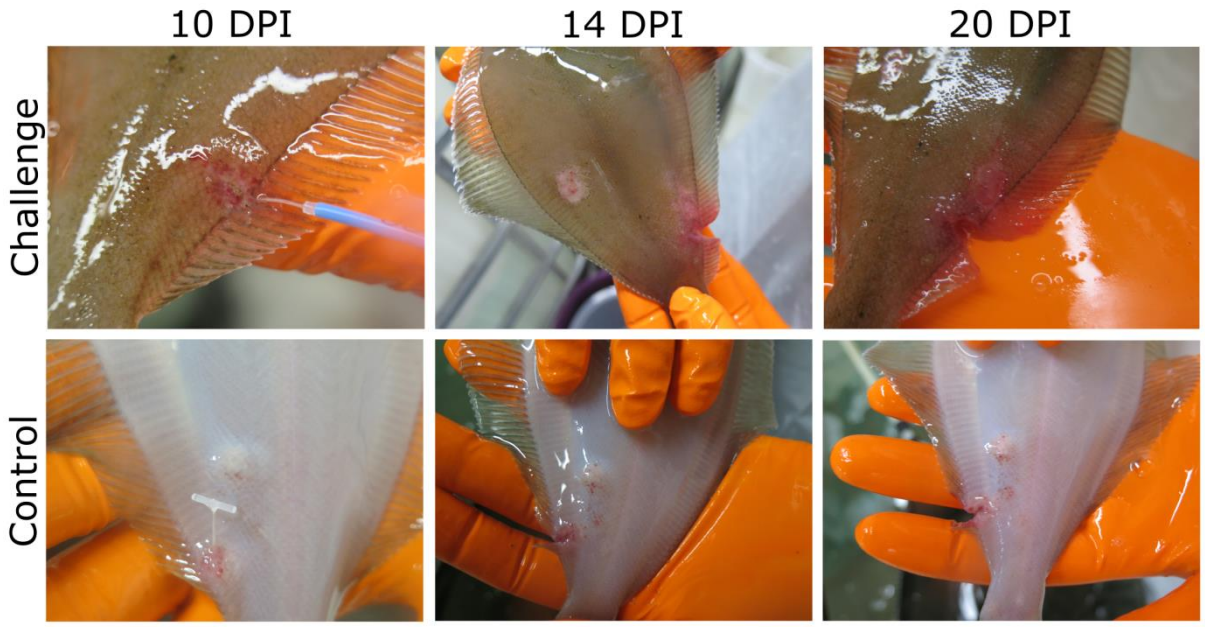


Figure 6.3: Examples of the evolution of the lesions that developed at the insertion site of the tag between 10 and 21 DPI. Examples are presented both for one challenged (above) and one control fish (below).

11-21 days post-inoculation (DPI)

In fish that died, generally a more severe lesion, based on the TUS, developed around the insertion site of the tag at 12 DPI, compared to fish that survived ($p = 0.0497$). The same observation was present at 14 DPI ($p = 0.0888$) and 16 DPI ($p = 0.0748$).

In control fish of replicate two, lesions remained absent or similar as before until the end of the trial. In control fish of replicate one, the lesions at the insertion site of the tag generally evolved to an ulceration over time (Figure 6.3). These lesions had a TUS of 1.82 ± 0.59 at 12 DPI which increased further up to 2.64 ± 0.79 at 21 DPI (Figure 6.4). In the challenged fish, the lesions developed to ulcerations with a TUS of 2.83 ± 0.22 at 12 DPI. The most severe ulcerations were observed at 14 DPI (3.29 ± 0.21). From then onwards, lesion development stagnated with TUS of 3.20 ± 0.27 at 21 DPI (Figure 6.4). At 12 and 16 DPI, a trend was observed indicating that lesions in the challenge group were more severe compared to the control group (Figure 6.3; 6.4). Between 18 and 21 DPI, no statistical significant difference between the challenge and control group was observed (Figure 6.4). *A. salmonicida* was isolated from the lesion at the insertion site of the tag in 9 out of 12 control fish and 9 out of 26 challenged fish at 21 DPI. Three cultures (two from samples of control and one from challenged fish) were pure; eight *A. salmonicida* isolates were co-occurring with one other colony type and the remaining isolates were part of an abundant culture.

6.4.3 Chronological changes in skin lesions outside predefined treatment zones

0-10 days post-inoculation (DPI)

Two challenged fish developed a small ulceration on 8 and 10 DPI. Both ulcerations started as pale zones on the P side and evolved into severe ulcerations of approximately 2.6 cm and 1 cm, respectively. Furthermore, small multifocal bulging lesions (1 – 5 mm) developed on the body after

treatment and/or inoculation in two challenged and one control fish around 8 or 10 DPI. A similar bulging lesion developed on the fins of one challenged fish. Five fish (one challenged fish, four control fish) developed a lesion on the lower jaw.

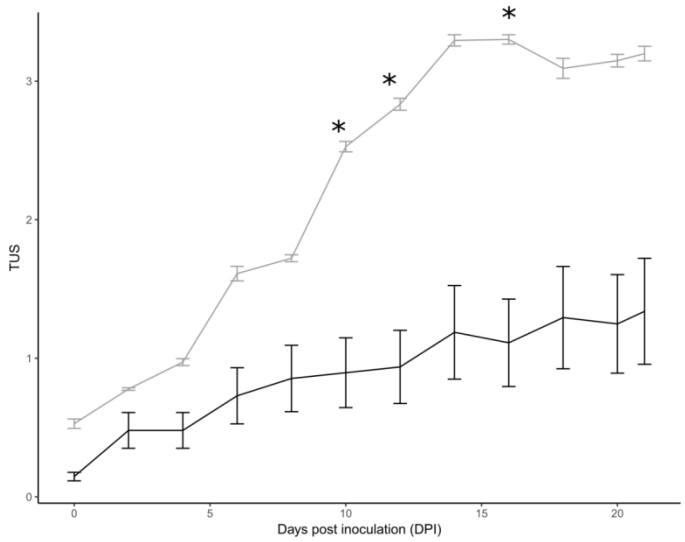


Figure 6.4: Overview of skin lesion assessment for lesions that developed at the insertion site of the tag. Average scores (± se) are depicted for the challenge (grey) and control group (black). Significant differences are indicated with two asterisk (**) and a trend is indicated with one asterisk (*). Only surviving fish were evaluated.

11-21 days post-inoculation (DPI)

Both fish which developed ulcerations outside the treatment zone died at 12 and 17 DPI, respectively. *A. salmonicida* was not isolated from the ulcerations. At 16 DPI, a challenged fish developed an ulceration at the P side, which aggravated until the end of the trial (21 DPI). Bacteriological sampling revealed the presence of an abundant culture of *A. salmonicida* in the ulceration.

The observed bulging lesions of the challenged fish that developed outside the treatment zone remained stable until the end of the experimental period. Almost all bulging lesions of the control fish were healed by 12 DPI. Furthermore, eight challenged fish and four control fish developed new bulging lesions between 12 and 18 DPI. In four (two of challenge and two of control group) out of 15 bulging lesions, *A. salmonicida* was isolated at 21 DPI.

One control fish with a lesion at the lower jaw died at 14 DPI. In the other four fish with lesions at the lower jaw, the lesions remained present between 11 and 21 DPI, and one challenged fish developed a new lesion at the lower jaw. *A. salmonicida* was isolated from two lesions at 21 DPI.

6.4.4 *VapA* gene sequencing

All isolates that were sequenced contained the *vap*A virulence gene. Maximum Likelihood (ML) tree analysis on the partial *vapA* genes from the selected *A. salmonicida* isolates (n = 18) recovered in the study identified all as A-layer type 15. However, two different sequences were obtained, originating from challenged fish (sequence 1) and from control fish (sequence 2). These were distinguishable by two nonsynonymous mutations within the gene fragment (417 nucleotides) examined. Sequence 1 was identical to that of the challenge strain (*A. salmonicida* strain 2CK). The partial *vapA* sequence of

the *A. salmonicida* strain from common dab in Denmark in 1999, identified previously as A-layer type 15 (Gulla *et al.*, 2019), received an intermediate position in the Maximum Likelihood tree, between sequences 1 and 2 (Figure 6.5).

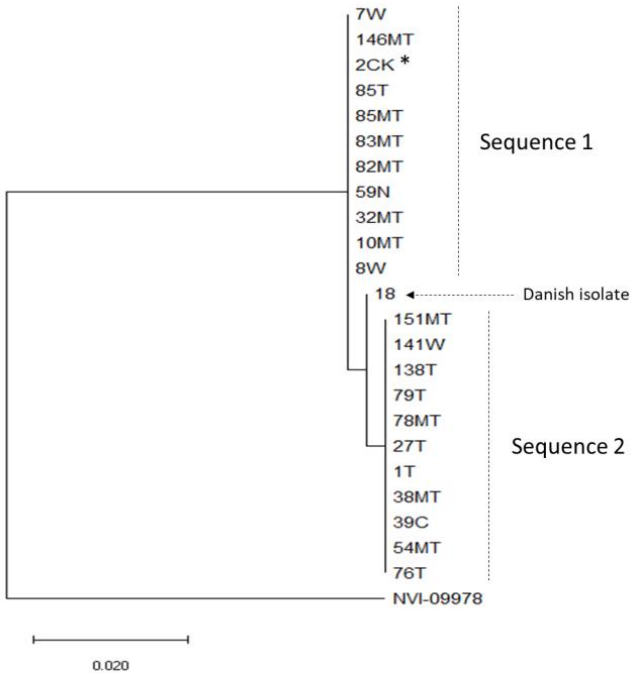


*Figure 6.5: Maximum Likelihood tree based on the partial vapA sequence from a selection of Aeromonas salmonicida isolates recovered in this study. An A-layer type 15 isolate (named "18") from common dab in Denmark in 1999 was also included for comparison, and the tree was rooted against strain NVI-09978 from Atlantic halibut in Norway, the closest non-type 15 A. salmonicida relative sequenced to date (Gulla et al., 2019). The two A-layer type 15 sequence variants found in the trial (Sequence 1 and 2) are distinguishable by two nonsynonymous mutations in the analyzed gene region (not shown). The isolate named "2CK" (indicated with *) is the isolate used for artificial inoculation of the challenge group and is clustered together with the isolates originating from challenged fish (sequence 1).*

6.5 Discussion

Both control and challenged fish developed ulcerations harboring *A. salmonicida*. However, sequencing of the *vapA* gene indicated that the *A. salmonicida* isolates retrieved from challenged and control fish were distinct, although the differences were minor (two nonsynonymous mutations). Sequence 1 was identical to that of the strain used for the challenge, *A. salmonicida* A-layer type 15 strain, 2CK. Moreover, the sequence of an older type 15 strain from common dab caught off the coast of Denmark connected, as an intermediate, the two variants found respectively from control and challenged fish, thus disputing a recent epidemiological link between these.

Since conventional, wild-caught animals were used, we find it most likely that some fish were already harboring *A. salmonicida* on the skin when entering the experimental facilities. As proven previously, *A. salmonicida* is known to establish asymptomatic infections and has been isolated in clinically healthy carrier fish (Austin & Austin, 2012; Bruno, 2015). Although it might be complicating factor in the interpretation of the research results, the presence of *A. salmonicida* is inherently connected to working with wild-caught fish. The value of common dab as an international biomarker for the health

of the ecosystem, urges the use of these wild-caught fish, despite the lack of information on their (microbiological and disease) history.

Both in challenged and sham treated control fish, *A. salmonicida* was isolated only in a minority of ulcerations. However, one should keep in mind that it may be difficult to re-isolate *A. salmonicida* from the diverse microbial communities commonly colonizing external lesions in various fish species (Austin & Austin, 2012). Hence, *A. salmonicida* is mostly recovered from only a small portion of diseased fish. The low re-isolation rate may be explained by the slow-growing phenotype of many *A. salmonicida* strains, often rendering them uncompetitive in growth media to faster growing opportunistic or environmental bacteria and/or the need for a certain amount of bacterial cells to enable growth in agar or in broth (Austin & Austin, 2012). Similar difficulties to isolate *A. salmonicida* from ulcerations in non-salmonid fish species were reported by Goodwin and Merry (2009). In future studies, these difficulties might be overcome by employing molecular methods for the identification of *A. salmonicida* in lesions. These methods have been used previously and have indicated that negative culture results were not always a good measure for the absence or presence of the pathogen in the lesion. Using a PCR technique seemed to be a more reliable to identify *A. salmonicida* in koi carp (*Cyprinus carpio*) ulcers (Goodwin & Merry, 2009). Nevertheless, our finding that *A. salmonicida* often occurred in pure or abundant cultures, may also be regarded as clinically relevant and points towards this agent playing a role in skin ulceration development. The extent to which and the adopted mechanism needs to be further elucidated. In following studies, to pinpoint the underlying pathogenesis, an infection with bioluminescent bacteria could be recommended combined with a sequential and controlled sampling during the development of the disease, as suggested by Bartkova *et al.* (2017) and Menanteau-Ledouble *et al.* (2011). Immunohistochemical staining can also be helpful to locate the bacteria during different stages of the disease.

The skin barrier is known to be one of the most important defense mechanisms of fish against various pathogenic micro-organisms or toxic compounds in the marine environment (Svendsen & Bøgwald, 1997). This study indicates that more ulcerations developed in the mechanically abraded zones compared to the chemically treated (mucus removed) or intact zones both in control and challenged fish. Therefore mechanical abrasion seems to enhance the ability of skin pathogenic bacteria to cause ulcerations and the intact skin may be regarded as an efficient protective barrier. In contrast, absence of the mucus layer, but with the presence of an intact skin, did not result in the development of more ulcerations. Hence, one might assume that the mucus layer is a less important barrier for an *A. salmonicida* infection. These results are consistent with previous studies in other fish species (Wiklund 1995; Svendsen & Bøgwald, 1997). When both skin and mucus barriers are intact (NT zone), no ulcerations developed, but remarkably, some ulcerations appeared outside the treatment zones, in macroscopically intact skin. This finding does not necessarily contradict the former reasoning. Indeed, as previously observed in common dab (Vercauteren *et al.*, 2019a) and flounder (Wiklund, 1995), it is possible that capture or handling of the fish may have caused small, unnoticed injuries which, during this experiment, were infected and colonized by pathogenic microorganisms.

The technique employed for mechanical treatment, removing scales and overlying epidermal layers, mimicked wounds inflicted by predators or fishing gear at sea (Davis & Ottmar, 2006) and has previously been successfully used by Vercauteren *et al.* (2019a) to demonstrate the role of *Vibrio*

tapetis in the development of skin ulcers in dab. The resulted abrasions leave the dermal fibronectin and collagen exposed which could facilitate the adherence of *A. salmonicida*. Although specific proteins involved in adherence were not determined, the *A. salmonicida* strain used for inoculation in the present study contained the *vapA* gene. This gene encodes a crystalline outer membrane protein known as the A-layer that is involved in cell adherence in pathogenic *A. salmonicida* strains (Dallaire-Dufresne *et al.*, 2014). Other previously reported factors involved in the adhesion of the pathogen to the fish tissue are LamB-like protein Omp48 (Dodsworth *et al.*, 1993), lipopolysaccharide O-antigen (Merino *et al.*, 1996; Reith *et al.*, 2008), flagella (Gavin *et al.*, 2002) and a type – IV pili (Kirov *et al.*, 1999). Adhesion may be followed by the further destruction and invasion of the fish tissue whereby the Type III secretion system might translocate toxins into the host cell cytoplasm (Burr *et al.*, 2005; Dallaire-Dufresne *et al.*, 2014). Furthermore, the hemolytic, leucocytolytic and proteolytic activity of extracellular proteins such as serine protease (aspA) (Whitby *et al.*, 1992), lipase (Dallaire-Dufresne *et al.*, 2014), cholesterol acyltransferase (GCAT) (Dallaire-Dufresne *et al.*, 2014) and metalloproteases (Reith *et al.*, 2008) might induce further damage to the fish tissues. Further research on the extracellular products produced by the A. *salmonicida* strain employed for challenge in the present study could potentially substantiate this hypothesis further.

The cause of death of the fish could not be established with certainty. One possible cause of death might be related to problems with maintaining the osmotic balance due to extensive lesions as the fish that died had developed larger and more severe skin ulcerations at the insertion site of the tag compared to the surviving animals. Furthermore, some of these fish also developed large ulcerations outside the treatment zones. Both might have increased the total area of damaged skin and, as reported before, damage covering as little as 10 % of the body surface could result in mortality (Carlisle & Roberts, 1977; Noga, 2000). Determination of osmolality in the blood could be useful to investigate this hypothesis. Alternatively, septicemia caused by *A. salmonicida* could also have resulted in the death of the fish as has previously reported in both salmonid and non-salmonid species (Wiklund & Dalsgaard, 1998; Austin & Austin, 2012). In the present study, myocarditis was histologically observed in three of the eleven fish that died, which might indicate a possible sepsis. However, gross or histological signs of septicemia were not present in other organs, nor was *A. salmonicida* isolated from any organ (liver, kidney or spleen) sampled. Some fish that died during the experiment were not sampled for bacteriological examination due to post-mortem decay.

Remarkably, development of ulcerative skin lesions and the frequent presence of *A. salmonicida,* in contrast to a lower re-isolation rate in mechanically treated zones, were observed at the insertion site of the tag. By inserting the tag, the skin barrier is impaired and the underlying muscle tissue partially exposed. As demonstrated by Ellis and co-workers (1981), *A. salmonicida* has a preference for adherence to and destruction of muscular tissue. The exposed muscle adjacent to the tag might therefore have favored the development of ulcerations induced by *A. salmonicida.* Furthermore, *A. salmonicida* was mainly isolated from lesions at 21 DPI and mortality was observed to be delayed in comparison to previous research using *V. tapetis*, albeit based on a different experimental population (Vercauteren *et al.*, 2019a). These observations might suggest that *A. salmonicida* has a role in later stages of ulceration development. This is in contrast with the results reported by Elliott and Scott (1980), who concluded that in goldfish, *A. salmonicida* is mostly present in early ulcerative lesions. More research is necessary to investigate this topic further.

In the present and previous study (Vercauteren *et al.*, 2019a), it was demonstrated that skin abrasion predisposes common dab for the development of skin ulcerations. However, other fish-related factors may also be involved, resulting in a presumably multifactorial cause (Mellergaard & Nielsen, 1997). In the present study, ulcerations developed more frequently and were more severe on the pigmented skin side of the fish. Remarkably, a similar skin side predisposition was observed in common dab challenged with *V. tapetis* (Vercauteren *et al.*, 2019a). The asymmetry of the flatfish is a fiercely discussed subject and both sides are macroscopically and presumably microscopically distinct. The most apparent microscopic differences are found in pigmentation, mucus-producing goblet cells (Faildé *et al.*, 2014) and scale morphology (Spinner *et al.*, 2016). Although we have good indications that the epidermal thickness of both sides is equal (data not shown), the reason for this predisposition hitherto remains unclear. Other fish-related characteristics such as age, sex and immune status were already assigned as possible predisposing factors in the development of skin ulcerations (Wiklund & Bylund, 1993; Mellergaard & Nielsen, 1997; Lang *et al.,* 1999). However, the set-up of the current study does not allow firm conclusions to be drawn on these factors.

In conclusion, based on the increased mortality and more severe lesion development in the challenged fish, *A. salmonicida* seems to be involved in the development of skin ulcerations in common dab although its relative importance and the underlying pathogenesis still needs to be elucidated. Prior skin damage seems to be an important predisposing factor in the development of skin ulcerations. One may further argue that the preference of *A. salmonicida* for adherence to and destruction of muscular tissue was substantiated by the development of lesions at the insertion site of the tag and re-isolation in later stages of the experiment. Indications that some of the wild-caught specimens in the control group most likely harbored prior (subclinical) *A. salmonicida* infections could conceivably explain the lack of difference in ulceration development between the challenged fish and sham treated control fish. Although this complicates the interpretation of the results, this research has added valuable information on the role of *A. salmonicida* in skin ulceration development in common dab.

6.6 Acknowledgements

The research was funded by the European Fisheries Fund (EVF – project VIS/15/A03/DIV), the Flemish Government and the Research Foundation – Flanders (FWO). This work makes use of the resources, facilities and/or services provided by UGent and Flanders Marine Institute as part of the Belgian contribution to EMBRC-ERIC. The funding bodies had no role in the study design, data collection, analysis or the writing process of the manuscript. Flanders Marine Institute (VLIZ), Flanders Research Institute of Agriculture, Fisheries and Food (ILVO) and the crew of the RV Simon Stevin are gratefully acknowledged for the help in the supply of fish. Dries Vandewoude, Wim Versteeg and Andre Cattrijsse are gratefully thanked for the daily monitoring of the fish during acclimatization at the Marine Station Ostend (MSO). The otolith lab of ILVO is thanked for the age determination of the fish. We acknowledge Christian Puttevils, Delphine Ameye and Joachim Christiaens of Ghent University for the outstanding technical assistance and/or help during the experiments.

Table 6.2: Mean Gross Ulceration score (GUS), Total affected area (TA) and Ulcerative area (UA) of the ulcerations that developed in the mechanically treated (MT-) zones. Averages are compared between (a) challenge and control fish and (b) between pigmented (P) and non-pigmented (NP) sides. An asterisk(*) indicates a trend, two asterisks(**) represent a significant difference between both groups or sides. Only fish that survived were included.

(a) Comparison between groups

	GUS			TA			UA		
DPI	Challenge		Control	Challenge		Control	Challenge		Control
0	4.79 ± 1.65		5.11 ± 1.12	1.96 ± 2.42		1.92 ± 0.35	0.00 ± 0.00	NA	0.00 ± 0.00
2	3.90 ± 2.32		4.59 ± 2.09	0.99 ± 0.74	**	1.43 ± 0.65	0.01 ± 0.06		0.05 ± 0.28
4	4.54 ± 3.26		5.02 ± 3.26	0.82 ± 0.70	*	1.00 ± 0.71	0.07 ± 0.14	**	0.26 ± 0.41
6	4.67 ± 3.63		5.02 ± 3.72	0.85 ± 0.80		0.99 ± 0.78	0.12 ± 0.27		0.21 ± 0.34
8	4.40 ± 3.56		4.59 ± 3.68	0.78 ± 0.73		0.92 ± 0.78	0.13 ± 0.29		0.19 ± 0.28
10	4.87 ± 3.41		4.11 ± 3.43	0.82 ± 0.69		0.87 ± 0.72	0.14 ± 0.29		0.14 ± 0.24
12	4.79 ± 3.49		3.96 ± 3.39	0.78 ± 0.67		0.87 ± 0.77	0.15 ± 0.30		0.11 ± 0.19
14	4.81 ± 3.63	*	3.76 ± 3.17	0.76 ± 0.63		0.79 ± 0.66	0.18 ± 0.32		0.06 ± 0.14
16	4.60 ± 3.34		3.96 ± 2.95	0.74 ± 0.56		0.76 ± 0.58	0.15 ± 0.29		0.07 ± 0.16
18	5.00 ± 3.40		4.11 ± 2.57	0.73 ± 0.55		0.75 ± 0.47	0.18 ± 0.33		0.04 ± 0.09
20	5.35 ± 3.12		4.33 ± 2.26	0.75 ± 0.52		0.69 ± 0.36	0.19 ± 0.35		0.02 ± 0.07
21	5.13 ± 3.15		4.02 ± 2.34	0.89 ± 0.63		0.83 ± 0.46	0.26 ± 0.49	*	0.03 ± 0.09

(b) Comparison between sides

	GUS			TA			UA		
DPI	P		NP	P		NP	P		NP
0	5.31 ± 0.71	*	4.57 ± 1.83	2.24 ± 2.38		0.80 ± 0.77	0.00 ± 0.00	NA	0.00 ± 0.00
2	5.57 ± 1.12	**	2.88 ± 2.26	1.59 ± 0.41	**	0.31 ± 0.46	0.02 ± 0.09		0.04 ± 0.26
4	7.12 ± 1.41	**	2.41 ± 2.83	1.50 ± 0.28	**	0.23 ± 0.39	0.27 ± 0.38	**	0.04 ± 0.15
6	7.57 ± 1.62	**	2.10 ± 3.03	1.60 ± 0.41	**	0.19 ± 0.33	0.30 ± 0.37	**	0.04 ± 0.12
8	7.35 ± 1.55	**	1.63 ± 2.67	1.50 ± 0.40	**	0.25 ± 0.35	0.28 ± 0.35	**	0.04 ± 0.12
10	7.29 ± 1.47	**	1.73 ± 2.40	1.45 ± 0.37	**	0.25 ± 0.49	0.22 ± 0.33	**	0.05 ± 0.15
12	7.18 ± 1.63	**	1.61 ± 2.35	1.39 ± 0.39	**	0.26 ± 0.41	0.20 ± 0.30	*	0.06 ± 0.18
14	7.04 ± 1.84	**	1.59 ± 2.34	1.29 ± 0.35	**	0.34 ± 0.44	0.16 ± 0.27		0.08 ± 0.24
16	6.61 ± 1.63	**	1.98 ± 2.57	1.16 ± 0.35	**	0.41 ± 0.43	0.16 ± 0.24		0.07 ± 0.23
18	6.73 ± 1.81	**	2.43 ± 2.47	1.07 ± 0.35	**	0.50 ± 0.4 3	0.15 ± 0.28		0.07 ± 0.23
20	6.69 ± 1.78	**	3.04 ± 2.39	0.94 ± 0.35	**	0.56 ± 0.52	0.14 ± 0.29		0.08 ± 0.26
21	6.29 ± 1.97	**	2.94 ± 2.59	1.16 ± 0.43	**	0.56 ± 0.52	0.21 ± 0.41		0.11 ± 0.36

NA: Not available

Part III

Integrative approach

Chapter 7

Report of a four-year survey: Pathogen-Fish-Environment

Based on: Vercauteren, M., Van Hoey, G., Decostere, A., Boyen, F., Ampe, B., Devriese, L., & Chiers, K. (2020). Influence of pathogens, fish-related and environmental factors on the development of skin ulcerations in wild common dab (*Limanda limanda*). Submitted in *Journal of Wildlife Diseases* in May 2020.

7.1 Abstract

Environmental changes or stressors can result in the development of diseases. Through regular fish disease surveys in the Belgian part of the North Sea, attention was drawn to a sudden increase of skin ulceration prevalence between 2011 and 2014. The involvement of two pathogenic bacteria was already suggested in previous research. During the surveys, information on prevalence, ulceration, bacteriology, fish-related factors (such as length, age and sex) and (spatial and temporal) environmental factors and fishing intensity were gathered. This detailed investigation is framed in the long-term monitoring, executed every spring-autumn from 2000 onwards. Ulcerations were observed in 1.3 % of investigated fish. Spatial and temporal differences were evident and highest prevalence was found in summer. *Vibrio* sp. was the dominant cultivated bacterial genus present in the lesions. Skin ulcerations appeared to be correlated with length and body condition of the fish, but also with temperature and pH of the sea water and fishing vessel density. This research therefore confirms the multifactorial aetiology of skin ulcerations in common dab and endorsed the effect we humans have on the marine ecosystem through human activities such as fishing and through climate change (with a change in temperature and pH).

7.2 Introduction

The Belgian part of the North Sea (BNS), as any other sea, is influenced by human activities such as shipping, fisheries, dumping of dredged material and input of hazardous material. Moreover, climate change is having a tremendous effect on the ocean affecting environmental factors such as temperature and pH of the sea water. Environmental changes or stressors can result in functional alterations in biochemistry and physiology of the blood and tissue of the fish, ultimately resulting in pathological changes and thus causing diseases (Sindermann, 1980; Giltrap et al., 2017). Fish diseases can thus be relevant as biomarker and used as integrative biological effect indicators, since changes in disease prevalence can provide information about the general changes in environmental quality. Hereby this contributes to the assessment of the health of the ecosystem (Snieszko, 1974; Giltrap et al., 2017). International standardized monitoring surveys were performed in various regions in the North Sea, guided by the International Council for the Exploration of the Sea (ICES). In the BNS, the Flanders Research Institute for Agriculture, Fisheries and Food (ILVO) has been responsible for the monitoring of fish diseases since 1985 (Devriese et al., 2015; data available from 2000 onwards). Through these regular fish disease surveys, attention was drawn to a sudden increase of skin ulceration prevalence in 2011, with a peak in 2013 (Figure 7.1) (Devriese et al., 2015). Generally, a decreasing trend in skin ulceration is observed since 2015 in all monitored areas (ICES, 2018). Nevertheless, together with the increase of skin ulceration prevalence in the BNS, a similar increase was observed in the German Bight with increases from 1.1 % to 2.0 % in summer and 0.4 % to 2.6 % in winter (ICES, 2012).

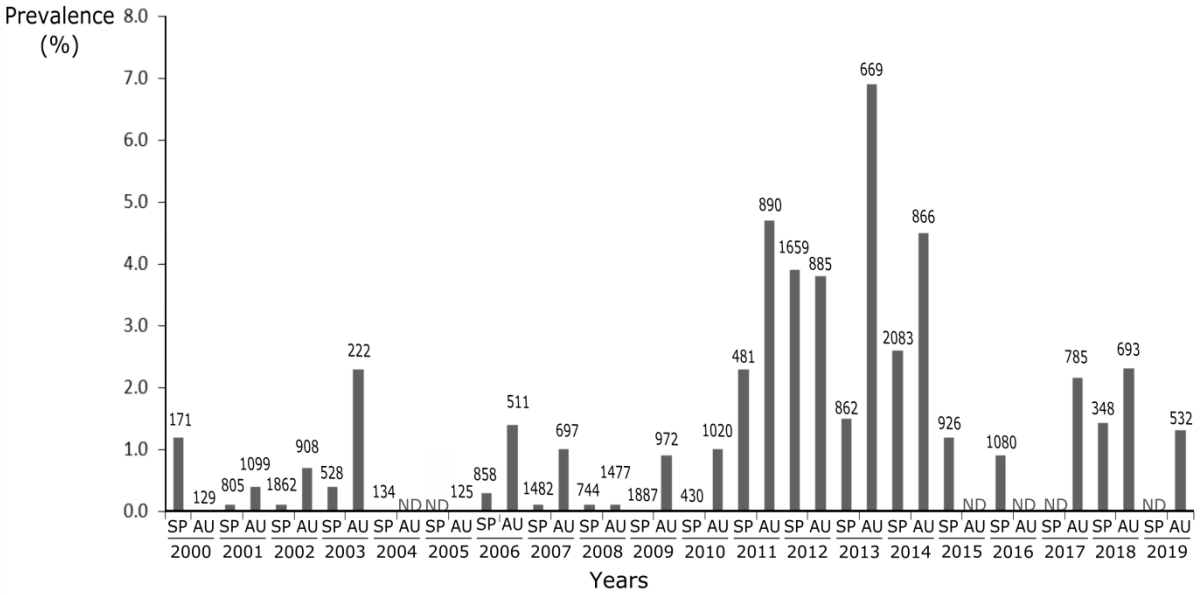


Figure 7.1: Evolution of average disease prevalence (calculated as the number of fish with skin ulcerations divided by the total number of fish caught) of skin ulcerations in common dab in the Belgian part of the North Sea. Data collected during the standardized two-yearly (spring: SP; autumn: AU) monitoring survey by the Flanders Research Institute for Agriculture, Fisheries and Food (ILVO) with RV Belgica. The numbers above each bar represent the number of fish caught. *ND: Prevalence not determined; survey was canceled.*

Moreover, in the Polish economic exclusive zone of the Baltic Sea, an increase in skin ulcerations in cod (*Gadus* spp.) was observed in 2011 with prevalence reaching 4.4 % (ICES, 2012). This sudden

change in disease prevalence preoccupied the scientific and sea-guarding communities (Devriese et al., 2015) and triggered scientists to perform an in depth data collection and analysis on the associations between the prevalence and possible risk-factors (environmental and anthropogenic).

Skin ulcerations are defined as a lesion where epidermis and basement membrane are missing resulting in exposure of the dermal and muscular tissue. The lesions often start by sloughing off the epidermal tissue and basement membrane resulting in open haemorrhagic lesions exposing dermal and muscular tissue (Wiklund and Bylund, 1993). For hitherto unknown reasons, flatfish are, based on previous reports and observational data, more vulnerable to the development of these lesions compared to round fish (Möller, 1981; Wiklund and Bylund, 1993). Since common dab (*Limanda limanda*) is a common flatfish and is included in the fish disease monitoring, we have focused on this species.

Inferences on the cause of these skin ulcerations are complex since a multifactorial aetiology can be suspected. Presumably, fish-related, environmental and pathogenic agents are playing a direct or indirect role in the development of skin ulcerations. In 2015, two bacterial agents, *Vibrio tapetis* and *Aeromonas salmonicida*, were isolated from active skin ulcerations in common dab (*Limanda limanda*) in the BNS (Vercauteren et al., 2018). In subsequent experimental studies (Vercauteren et al., 2019), it was found that *V. tapetis* and *A. salmonicida* are able to cause these ulcerations but skin abrasions seemed to be a major contributing factor in the development of skin ulcerations, indicating the multifactorial causality of skin ulcerations (Vercauteren et al., 2019; Vercauteren et al., 2020). These results were in agreement with Snieszko (1974) who stated that "an *overt infectious disease occurs when a susceptible host is exposed to a virulent pathogen under proper environmental conditions*".

In an attempt to grasp the complexity of the aetiology of skin ulceration, a multidisciplinary survey has been conducted to quantify its geographic spread, prevalence rates among different locations and involvement of bacteria. Associations between the fish-related characteristics (length, weight, age,…), spatial and temporal patterns, environmental and anthropogenic parameters and the prevalence of skin ulcerations were studied based on data collected in the BNS on two-monthly surveys from 2016 until 2019.

7.3 Materials and methods

7.3.1 Study area and sampling sites

This study was carried out in the BNS (3457 km²), part of the Southern North Sea and located in the IVc ICES statistical area (ICES, 2019a; Marineregions, 2019). Eight sampling sites were chosen based on their scattered geographic distribution in the BNS (Figure 7.2). The sampling sites were monitored every two months between 2016 and 2019 on board of the Research Vessel (RV) Simon Stevin equipped with a beam trawl (3 m; mesh size: 22 mm) and a Conductivity-Temperature-Depth (CTD)-probe (Seabird 19plusV2, Sea Bird Electronics, USA). Fish were caught using a 3 m beam trawl that was towed during approximately 20 minutes with a speed of 3 to 4 knots. The sampling was carried out in accordance with the approved guidelines and legislation in force with regard to animal welfare.

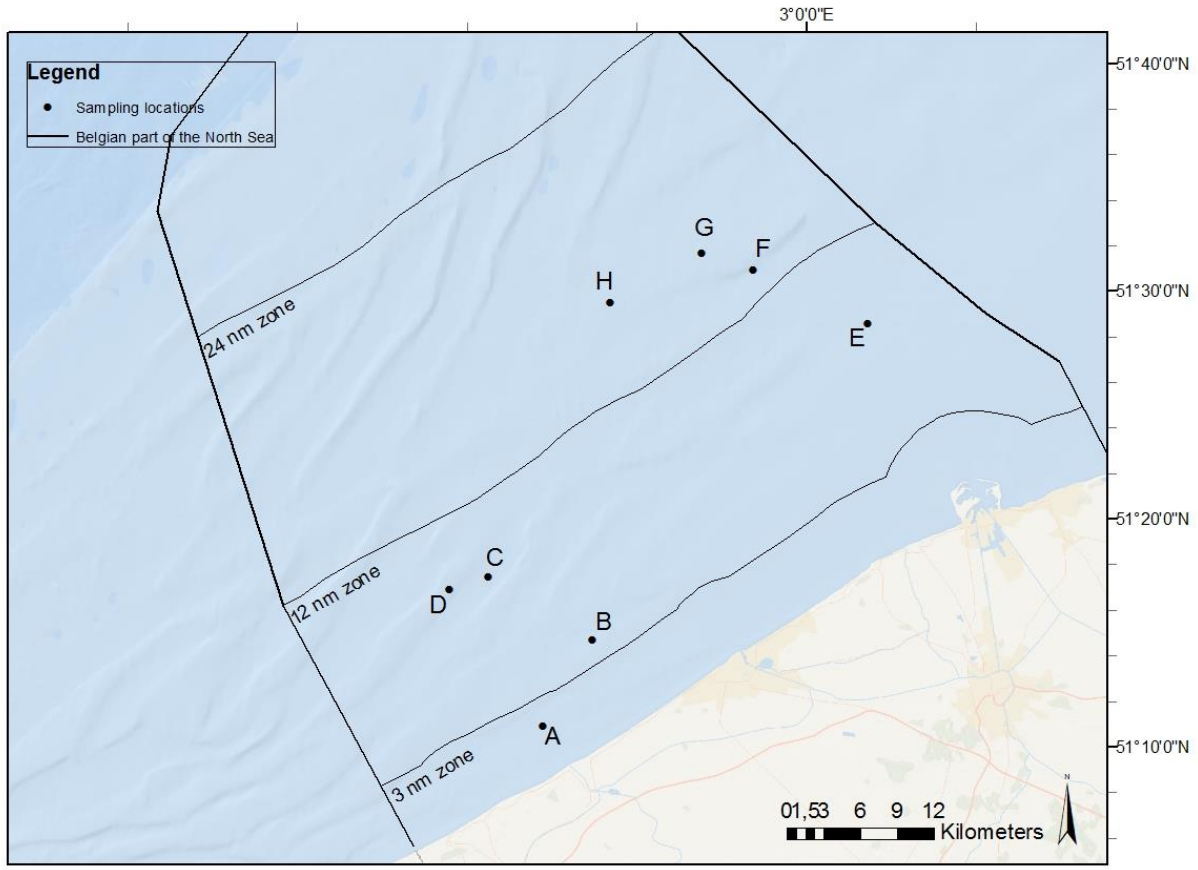


Figure 7.2: Map of the survey locations in the Belgian part of the North Sea that were monitored during this research. The outline of the Belgian part of the North Sea is provided together with the 3, 12 and 24 nautical mile (nm) zones.

7.3.2 Fish surveys – disease prevalence

The total catch was sorted and common dab were subjected to further investigation. Each common dab was examined thoroughly for the presence of externally visible diseases. Since this study focusses on skin ulcerations, fish with skeletal deformities, hyper- or hypopigmentation, (healed) lesions, and papilloma-like lesions were excluded for further analyses, but information regarding these abnormalities is included in Supplementary file 2.

Fish with skin ulcerations were investigated thoroughly. The ulcerations were recognized as clearly delineated haemorrhagic lesions with a rim of cutaneous petechial haemorrhages surrounding the lesion, in compliance with the guidelines compiled by ICES (Bucke et al., 1996) and with our previous research (Vercauteren et al., 2018). The prevalence of the disease was calculated as the number of fish with skin ulcerations divided by the total number of dab caught at each sampling site. Catch per unit effort (CPUE), i.e. the number of dab caught per hour of trawl time for each sampling site and each survey was used as a proxy for population density (Mellergaard and Nielsen, 1995).

7.3.3 Examination of ulcerations

On board, fish with ulcerations were humanely sacrificed using an overdose of benzocaine (Ethyl 4-aminobenzoate, final concentration 200 mg L^{-1} of seawater; Merck, Germany) or tricain

methanosulfonate (MS-222; 500 mg L^{-1}; Sigma Aldrich N.V., Belgium) and subjected to an examination. Following characteristics of the ulcerations were noted: location on the body, stage (acute/healing) and intensity (i.e. the number of ulcerations per fish). A swab from the ulcer was taken for bacteriological examination. Sagittal otoliths were collected to determine the age using the method described by the ICES workgroup WKARDAB2 (ICES, 2016). A full necropsy was performed to inspect the internal organs on macroscopically visible abnormalities. The stomach was removed in a subset of fish and later on analysed for the presence of microplastics (Supplementary file 3). Per 10 fish with ulcerations for each haul, one control fish without externally visible diseases was sacrificed and investigated with the same protocol. Later on, in the lab, the area (cm^2) of the ulcerations was measured using scientific image software (Image J, version 1.4) based on photographs.

7.3.4 Bacteriological examination

Bacteriological swabs of the lesions of 48 fish were immediately after collection inoculated on Marine Agar (Scharlau Microbiology, Spain), Shieh agar supplemented with 1.5 % NaCl (Shieh and Maclean, 1975; Shieh, 1980; Song et al., 1988) and Columbia agar with 5 % sheep blood (blood agar; PB5039A, Oxoid, United Kingdom). Back in the lab, agar plates were aerobically incubated for a week at 16 ± 1 °C, isolates were purified if possible and pure cultures were frozen for further analysis. Cultures in which more than five colony types (without clear dominant type) were present were not further analysed. Frozen samples were thawed later on and plated on their original medium type. After 24 h of growth, colonies were identified with the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) technique, using the direct transfer method. Each isolate was spotted on a polished steel target plate in triplicate, air dried, covered with 1 µl alpha-cyano-4-hydroxycinnamic acid matrix (Bruker Daltonics, Bremen, Germany) and processed with an Autoflex III smartbeam MALDI-TOF MS (Bruker Daltonik GmbH, Bremen, Germany) and commercial software using standard settings (flexControl 1.4, version 3.4., Bruker Daltonik GmbH, Bremen, Germany). The spectra were analyzed using MBT Compass version 4.1 (Bruker Daltonik GmbH, Bremen, Germany) that included a reference database of 7926 different bacterial entries (including *V. tapetis* and *A. salmonicida*), using standard settings. A (log) score value between 1.7 and 2.0 represents identification at species level at low confidence, and a (log) score value ≥ 2.0 represents identification at species level at high confidence.

7.3.5 Fish-related factors

Total length (L), weight (W) and sex were determined for each common dab that was caught. The Fulton body condition factor was calculated based on the weight and length ($K = 100 * (W / L^3)$) (Fulton, 1904). Due to practical restrictions, fish smaller than 10 cm (n = 1071) were not weighed separately, although they were individually inspected for the presence of diseases. These fish were excluded from the descriptive and statistical analysis.

7.3.6 Environmental factors

On board of the RV Simon Stevin, sea water temperature, depth, turbidity, oxygen saturation and salinity of the water were measured just above the seabed using the CTD-probe. The pH of the water collected at the seabed was measured (Hanna Instruments, Belgium).

Data on anthropogenic activities on each sampling location such as dredging, offshore energy and maritime transport was extracted from Kustportaal, a digital data portal for marine data (Kustportaal, 2019). The unsmoothed Atlantic Multidecadal Oscillation (AMO)-index, calculated at the Physical

Sciences Division of the National Oceanic and Atmospheric Administration Earth System Research Laboratory, was downloaded and basically represents an index of the North Atlantic sea surface temperatures on a monthly interval (ESLR-PSD, 2019). Information and data regarding pollution (concentration of heavy metals and polychloorbifenyl (PCB) in the sediment; granular fraction < 63 µm) was provided by ILVO. Degree of pollution was assessed by using the five defined pollution cluster zones in the BNS with each a different set of pollutants (Lagring et al., 2018). Distance from the shore was calculated perpendicular to the shore based on the coordinates of the sampling points. Information regarding substrate on the seabed was downloaded from the online database of the European Marine Observation and Data Network (EMODnet) – Seabed Habitats (EMODnet, 2019a). Mean shipping density (various types of vessels) and mean fishing vessel density (hours per square kilometre per month) were subtracted from the EMODnet Human Activities data portal (EMODnet, 2019b). The fishing vessel density is available per month and both fishing vessel density in the month of the survey and one month before were used.

7.3.7 Data analysis – Determination of risk-factors linked to ulceration development

Determination of risk-factors linked to ulceration development was studied using a linear regression model (LRM). Collinearity between variables was tested beforehand using graphic interpretation and the Pearson correlation coefficient. If collinearity was observed, only one of the variables (the one considered to be biologically the most relevant) was introduced in the model, to avoid misinterpretation of the results. Collinearity was observed between: length and weight, length and age, depth and distance to shore, season and sea water temperature, and season and fishing intensity.

Table 7.1: Environmental factors that were included in the LRM as explanatory variables.

Spatial and temporal (environmental) factors
Catch per Unit of Effort (CPUE)
Pollution
Sediment type
Anthropogenic activities
Depth
Year
Sea water temperature
Oxygen saturation
Salinity
pH
Turbidity
AMO – Atlantic Multidecadal Oscillation
Fishing vessel density
Fishing vessel density - 1 month before survey
General vessel density (all vessel types)

Stepwise model selection using a LRM was performed using forward selection by adding the factor with lowest p-value in each step. The model was considered to be final when addition of extra factors did not result in improvement of the model (based on Akaike Information Criterion) or significance of

newly added factors. The presence or absence of ulcerations was used as response variable. Fish-related and environmental factors that were used as explanatory variables are listed in Table 7.1. A mixed logistic regression model (MLRM) was tested using sampling location as random intercept; however, this resulted in singular fit of the data. Despite the overfitting, the results of the MLRM and LRM were similar. All statistical analyses were performed using RStudio.

7.4 Results

7.4.1 Catch data

A total of 3999 common dab (> 10 cm) were caught. Fish had an average length of 17.2 ± 3.5 cm with a maximum size of 31 cm. The average weight was 55.4 ± 36.5 g. The average body condition (K) of the fish was 0.99 ± 0.26 and the fish had an age between 1 and 7 years. In 3819 (95 %) common dab, sex was determined; most fish were females (59 %). Similar sex ratios were observed amongst sampling sites B, C, D, E and H. In site A, relatively more females were present with a ratio of 33 % Males / 67 % Females. In site F, the sex ratio approached 50/50 % and in site G the ratio was reversed with 62 % male fish and 38 % female fish. Except for the latter location, females always dominated. Female fish had an average length of 17.8 ± 3.7 cm and male fish of 16.3 ± 2.9 cm. No clear difference in length could be observed amongst the different sampling sites, although female fish were on average larger than the males at every site. In each of the sampling sites, between 120 and 800 dab were sampled over the study period.

7.4.2 Overall ulceration prevalence

Of the 3999 examined fish (> 10 cm), 52 fish (1.3 %) had a skin ulcer. Maximum ulceration prevalence observed per location per sampling when sufficient (> 50) fish were caught was 16 %.

Malpigmentation (1.8 %) and skin ulcerations (1.3 %) were the most prevalent followed by skeletal deformities (0.7 %), healed lesions (0.4 %), active lesions (0.3 %) and papilloma-like lesions (0.2 %) (Supplementary file 2).

7.4.3 Examination of ulcerations

Of the 52 fish with ulcerations, 27 % showed signs of healing in the ulceration recognized by a white, raised edge around the lesion and little to no haemorrhage in the surrounding tissue. Even though they were healing, the centre of the lesions was still ulcerated. No distinction between active and healing ulcerations is made in the analysis in this study.

Lesion surface ranged between 0.03 cm^2 and 1.8 cm^2 with an average size of 0.4 ± 0.5 cm^2. The highest number of ulcerations per fish was five, which was observed in two fish. In the majority of fish (75 %), only one ulcer was present. Ulcerations were present at either the pigmented or non-pigmented side, except in one fish with ulcerations on both sides. Ulcerations were mainly located on the non-pigmented side (65 %) and to a lesser extent on the pigmented side (33 %). The same distribution was found in males and females, with slightly different ratios. The ulcerations were found not be restricted to a specific zone on the body of the fish. In total 52 % of the ulcerations

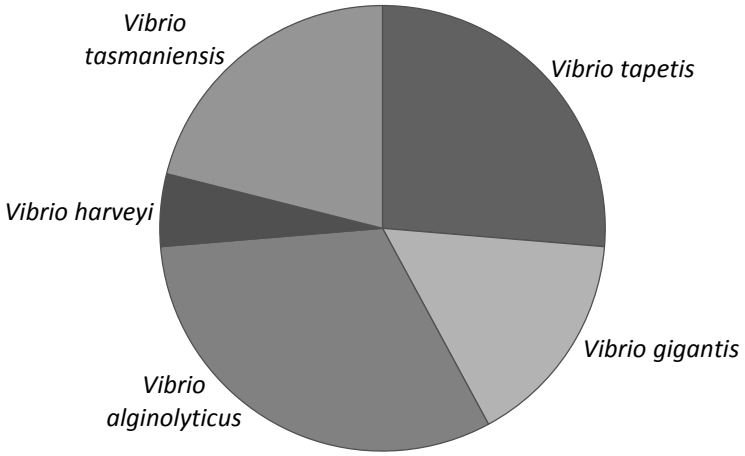


Figure 7.3: Visualization of the amount of skin ulcerations containing Vibrio sp. whereby V. alginolyticus and V. tapetis are the most commonly isolated species.

were found on the ventral side (independent of pigmented or non-pigmented side). In female fish 58 % of the ulcerations were found on the ventral side and 24 % in the close proximity of the lateral line. In males 40 % of the ulcerations were found on the ventral side and 36 % in close proximity of the lateral line, independent of the side. No consistent abnormalities in the internal organs were observed in fish with skin ulcerations.

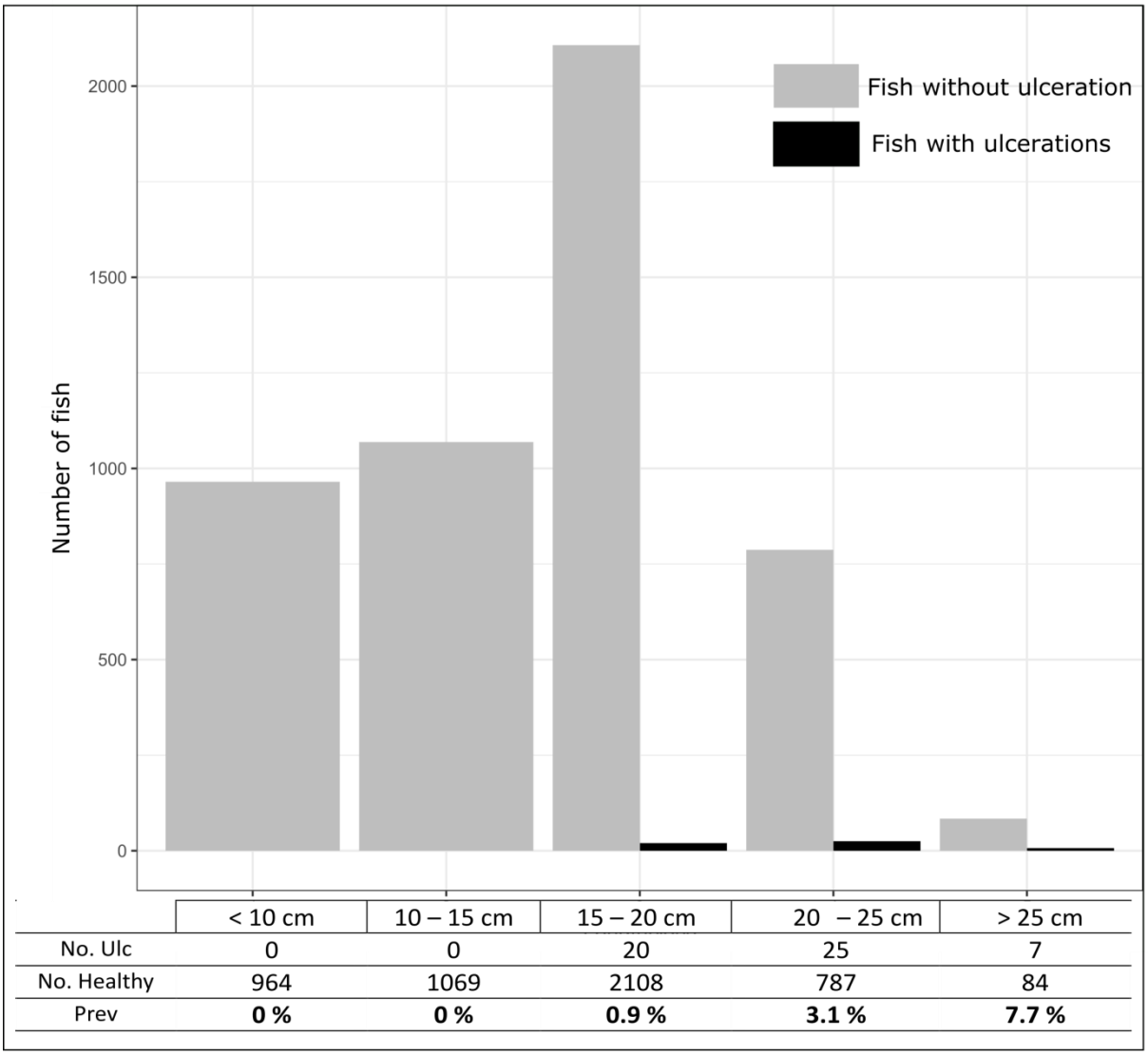
7.4.4 Bacteriological examination

Bacteriological results indicated that *Vibrio* sp. was the most commonly cultivated bacterial genus in the skin ulcerations, present in 81 % of fish with skin ulcerations. Remaining identified genera are *Bacillus* sp. (14 %), *Psychrobacter* sp. (14 %) and *Actinobacter* sp. (10 %). Other genera were present in less than 5% of the ulcerations. Amongst the *Vibrio* sp., *V. alginolyticus* was the most prevalent

with presence in 27 % of the ulcerations. The second most prevalent *Vibrio* species was *V. tapetis*, present in 23 % of the lesions, followed by *V. tasmaniensis* (18 %) (Figure 7.3). Bacterial species were mainly found as dominant colony type in a poly culture, although all three species (V. *alginolyticus*, *V. tapetis* and *V. tasmaniensis*) were each found once in a pure culture. *Aeromonas* sp. was identified in one lesion with low confidence score (> 1.7; < 2.0) for species identification.

7.4.5 Fish-related differences in ulceration prevalence

Fish with ulcerations were on average 20.5 ± 2.9 cm in length and healthy fish on average 17.2 ± 3.5 cm. The highest prevalence of skin ulcerations was found in fish larger than 25 cm (7.7 %) and no ulcerations were found in fish between 10 and 15 cm (Figure 7.4). The weight of fish (in the same length class (15 – 28 cm)) with and without ulcerations was 77.9 ± 33.4 gr and 66.7 ± 33.6 gr, respectively. Fish with ulcerations had a body condition of 0.9 ± 0.1. Mean body condition of healthy fish (from the same length class as fish with ulcerations (15-28 cm)) was 1.0 ± 0.2. Male and female fish showed skin ulceration prevalence of 1.3 % and 1.4 %, respectively. Male fish had on average 1.3 ± 0.9 ulcerations per fish and females 1.5 ± 0.9 ulcerations per fish. Age was determined for 45 fish



	< 10 cm	10 – 15 cm	15 – 20 cm	20 – 25 cm	> 25 cm
No. Ulc	0	0	20	25	7
No. Healthy	964	1069	2108	787	84
Prev	**0 %**	**0 %**	**0.9 %**	**3.1 %**	**7.7 %**

Figure 7.4: Prevalence of fish with ulcerations, calculated by the ratio of the number of fish with ulcerations (No. Ulc, Black) divided by the number of fish without an ulceration (No. Healthy; Grey) in each length class.

with skin ulcerations and control fish (n = 35). Fish with skin ulcerations were on average 3.5 ± 1.2 years old and control fish 2.9 ± 1.0 year.

7.4.6 Spatial and temporal difference in ulceration prevalence

Disease prevalence of fish from different sampling locations varied between 0.6 % and 2.8 %. Station C and E contained 50 % of all skin ulcerations found, resulting in prevalence of 2.1 and 2.8 %, respectively (Figure 7.5). Since there were no major differences observed in sex ratio or average length at the sampling sites, direct comparison of disease prevalence between sites is possible.

The highest prevalence was found in summer with in total 3 % of the fish affected (Figure 7.6). In spring 1.4 % of the fish showed skin ulcerations (Figure 7.6). This pattern was roughly similar in 2017, 2018 and 2019 but in 2016, more ulcers were found in winter. Some slight variations were observed between sampling sites; however, spring and summer have highest prevalence in most cases. The reverse was observed in the long term monitoring (Figure 7.1), where the prevalence was always higher in autumn compared to spring.

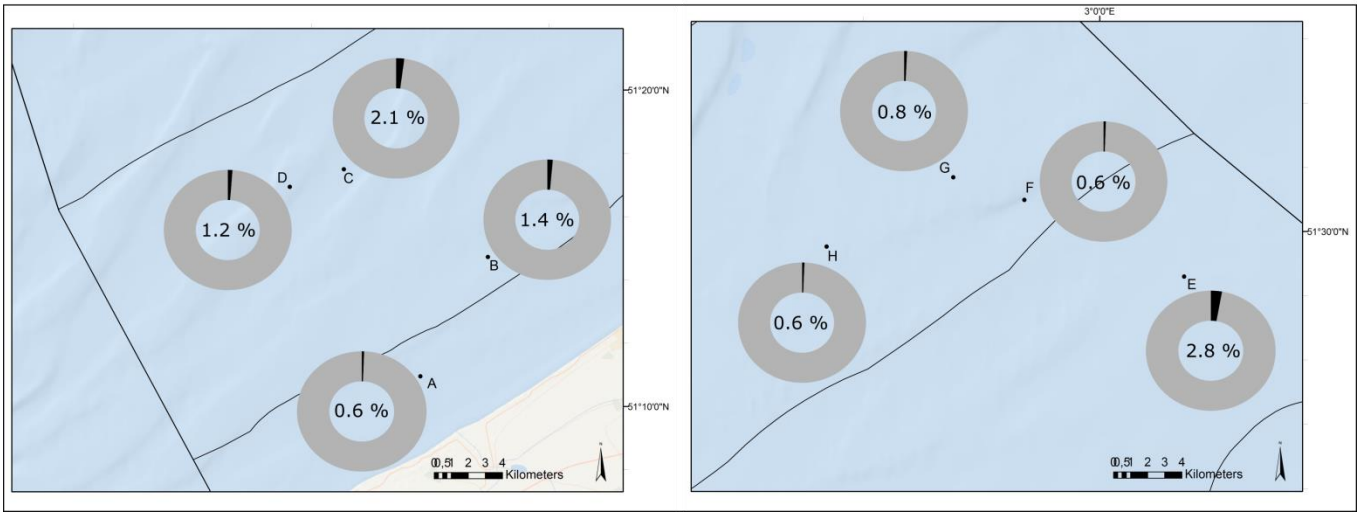


Figure 7.5: Spatial differences in skin ulceration prevalence

7.4.7 Determination of risk factors linked to ulceration development

Analyses on possible related risk factors in the development of skin ulcerations were only studied with fish larger than 15 cm, since no skin ulcerations were observed in smaller fish.

The prevalence of ulcerations was, according to the LRM, significantly associated with length ($p < 0.0001$) and body condition ($p < 0.0001$) of the fish (Table 7.2). Sex did not show a significant association with the presence of a skin ulceration ($p = 0.7050$). Most of the environmental parameters, such as depth, pollution, stock density and sediment type did not show an association with skin ulcerations ($p > 0.05$). It was found that both sea water temperature ($p = 0.0005$) and pH ($p = 0.0308$) show a significant positive correlation with the presence of skin ulceration (Table 7.2). Other seasonally fluctuating factors such as salinity, oxygen saturation, turbidity and the AMO-index did not show a significant association with skin ulceration development. Fishing vessel density in the month of the survey showed both spatial differences and seasonal variations and was positive associated with skin ulcerations ($p = 0.0173$) (Table 7.2). Fishing density one month before the survey did not affect ulceration development and neither did the general shipping density (various types of vessels).

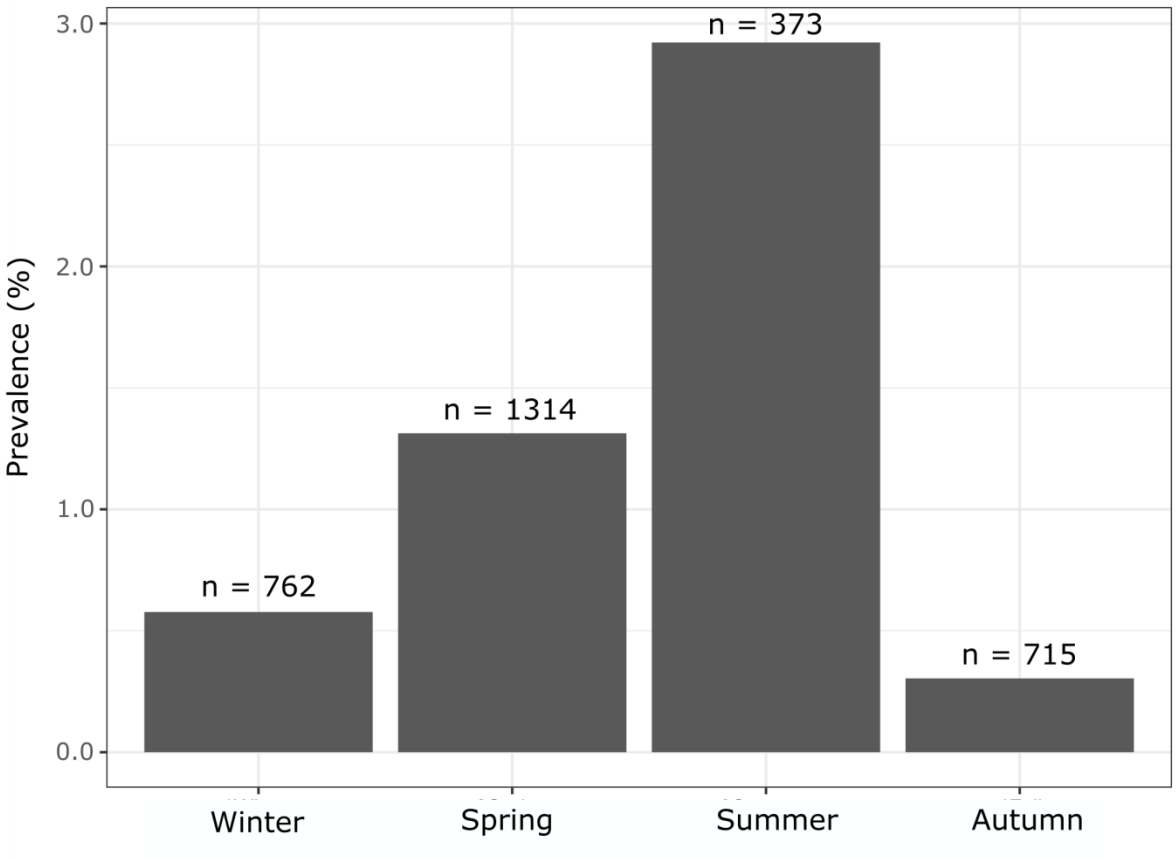


Figure 7.6: Seasonal variation in prevalence of skin ulcerations.The numbers indicate the number of fish per season.

7.5 Discussion

According to recent research, the health status of the North Sea has improved since the 1980's (Lang et al., 2017). Nevertheless, sudden and climate driven changes, new stressful stimuli and emerging contaminants, amongst others, occur. Examples are the sudden increase in skin ulceration prevalence observed along the west coast of Denmark in 1989 (Mellergaard and Nielsen, 1997) or the sudden increased ulceration prevalence in the BNS in 2011 (Figure 7.1; Devriese et al., 2015). Formulating inferences on causality of sudden changes is not straightforward and the presumable multifactorial aetiology of skin ulcerations acts as a complicating factor. Therefore, a detailed, multidisciplinary, four-year survey implementing pathogenic agents, fish-related factors, temporal and spatial environmental factors, was needed to get more insights in the correlated risk factors and the presence of skin ulcerations. In this small-scale study, variations in prevalence were observed and could be explained by both fish-related characteristics, environmental factors (sea water temperature and pH) and fishery activity (see further). This confirms the presumed multifactorial aetiology of skin ulcerations. This is to our knowledge the first integrative and comprehensive study on the aetiology of skin ulcerations in common dab in the BNS. The results found in this study are believed to be highly relevant for all marine ecosystems worldwide under influence of climate change and changing anthropogenic stressors.

The prevalence found in this study (approximately 1.3 %) was in line with the values of the long-term 'external fish diseases' monitoring of the same period (only autumn) (1.9 %) (Figure 7.1). Both were lower than the skin ulceration prevalence values (5.0 %) as observed in 2011 - 2014, a period that seems to be an exception on the normal pattern. Nevertheless, the recorded prevalence now is still slightly higher than observed before 2011 (1.0 %), which need further attention and stresses the importance of executing long-term monitoring for fish diseases. Although the prevalence is lower compared to prevalence in 2011 - 2014, skin ulcerations are still the second most prevalent encountered lesions during the surveys (Supplementary file 2). Previously reported prevalence of skin ulcerations in larger parts of the North Sea have been ranging from lower than 0.6 % (Mellergaard an Nielsen, 1995) up to 25 % (Dethlefsen, 1990). Vethaak and colleagues (2009) reported a general upward trend in skin ulcers in dab between 1990 and 2005. In other regions, such as the Dogger Bank a previous hot-spot for skin ulcerations, the prevalence has dropped from 22 % prevalence to 1.3 % nowadays (Lang et al., 2017; ICES, 2018).

7.5.1 Fish related characteristics

This study confirms that the choice of investigating skin ulceration for regulator monitoring purpose (cf ICES guidelines for fish disease surveys), on dab greater than 15 cm is correct, as none of the fish smaller than 15 cm showed skin ulceration. The length of the fish showed a positive correlation with the presence of skin ulcerations (Figure 7.4), suggesting that larger fish are more likely to develop ulcerations. This is in accordance with previously reported results in common dab (Mellergaard and Nielsen, 1995; Mellergaard and Nielsen 1997; Vethaak et al., 2009) and flounder (Wiklund and Bylund 1993). Wiklund and Bylund (1993) noticed that the length of the smallest flounder found with a skin ulcer correlated with the length of sexual maturity, presumably related to behavioral and anatomical changes. In common dab, male fish reach sexual maturity at 1 year (~ 11 cm) and females between 2 and 3 year (~ 14 cm) (Rijnsdorp et al. , 1992). Therefore, one might assume that the chance of developing an ulcer might increase after reaching sexual maturity in common dab as well (Wiklund and Bylund 1993). However, this hypothesis would not explain the increasing ulceration prevalence with length.

Furthermore, skin ulcerations were negatively correlated with the body condition, reflecting the nutritional stage of the fish. The association between body condition and skin ulcerations has not been consistently reported in literature, Mellergaard and Nielsen (1995) reported no correlation between skin ulcerations and the body condition and Mellergaard and Nielsen (1997) reported a negative correlation just as Vethaak and colleagues. (2011). Important to note is that this is merely an observation and this correlation can be true in both directions. Fish with lower body condition might be more susceptible to diseases such as in skin ulcerations. However, the presence of an ulcer can cause a depression of the body condition, i.e. by impeding the osmotic balance (Mellergaard and Nielsen, 1997). Further experimental research might elucidate this dual association and ascertain true causality.

In this study, sex was not observed to be associated with the development of skin ulcerations. No real consensus is present in existing literature on the association between sex and the development of skin ulcerations (Mellergaard and Nielsen, 1995; Mellergaard and Nielsen, 1997; Dethlefsen et al., 2000). Where sex-related differences were found, they were mostly linked to difference in size, since female fish are usually slightly larger compared to males (Mellergaard and Nielsen, 1997). Other

explanations are different migratory patterns and differences in growth and physiology (Dethlefsen et al., 2000).

7.5.2 Bacteriological research

Based on the bacteriological examination, *Vibrio* spp. seem to be important pathogenic agents that are regularly isolated from these lesions. Not only *V. tapetis*, which was described previously as an important factor in skin ulceration development (Vercauteren et al., 2018; 2019), but also *V. alginolyticus* seemed abundantly present. *V. alginolyticus* is reported in marine and estuarine environments (Austin and Austin, 2015). Outbreaks of vibriosis caused by *V. alginolyticus* have affected several aquaculture systems for marine species such as sea bass (*Dicentrarchus labrax*) (Khalil et al., 2019), seabream (*Sparus aurata*) (Zorrilla et al., 2013; Khalil et al., 2019), turbot (*Scophthalmus maximus*) (Austin et al., 1993), and grouper (*Epinephelus malabaricus)* (Lee, 1995). Clinical signs linked with infection are haemorrhagic ulceration, protrusion of the eyes, tail rot and mortalities (Austin and Austin, 2015; Khalil et al., 2019). *Vibrio* spp. have progressively been reported in the North Sea whereby the geographic spread could be correlated with biotic and abiotic factors such as sea water temperature, salinity and phytoplankton composition (Oberbeckmann et al., 2012).

7.5.3 Environmental characteristics

Temporal variation in disease prevalence was observed in all years with the highest prevalence mainly observed in the spring and summer. This is in contrast with previous studies that also reported temporal differences with peaks mainly in autumn (Wiklund and Bylund 1993; Devriese et al., 2015). The temporal pattern appeared to be mainly driven by seasonal changes in sea water temperature and pH, with increase in both factors resulting in higher chance on the development of skin ulcerations. Both environmental factors are assumed to change further due to climate change (WWF, 2018). Importantly, this is assumed to be an indirect effect that can act both on the pathogens involved and on the susceptibility of the fish to fight infection. Correlation between skin ulceration development and rising sea water temperature have been recently described in various species in the Galapagos following El Niño events (Lamb et al., 2018). Both sea water temperature and pH are expected to be changing slowly and might have a more seasonal effect (Mellergaard and Nielsen, 1997). In previous research, oxygen depletion did affect the development of epidermal papilloma and lymphocystis, however, the development of skin ulcerations was not affected (Mellergaard and Nielsen, 1995; Mellergaard and Nielsen, 1997), as also observed in the present research. Furthermore, although the general reported decrease of skin ulcerations has been linked to an improvement of the water quality, pollution could not directly be associated with the development of skin ulcerations based on the data gathered in this study. The study of Vethaak et al. (2011), was able to pinpoint a role of pollution in the development of skin ulcerations in flounder. However, the raised levels of contaminants were largely unrelated to concentrations in the sediment, changes in bioavailability or biotransformation or changes in redistribution of these substances might explain the observed effects (Vethaak et al., 2011).

Importantly, since the method used in this study does not allow measuring sudden changes (changes that occurred in-between two monitoring campaigns) in environmental parameters; the absence of associations between an environmental factor and the presence of skin ulcerations cannot exclude an important influence of short term fluctuations. For salinity, such fluctuations were described to be important in skin ulceration development (Mellergaard and Nielsen, 1997; Vethaak *et al*., 2011; Vethaak, 2013). More regular measurements of environmental parameters would offer the

possibility to include theses sudden changes in the model. Nevertheless, this short-term study revealed some important environmental risk factors that need to be considered for the long-term monitoring in any area (e.g. long-term pH data is not available).

7.5.4 Fishing intensity

One important result of this study was the association between fishing vessel density and the presence of skin ulcerations. The fishing vessel density map was based on the Automatic Identification System (AIS) and not on Vessel Monitoring System (VMS) which might result in an underestimation of vessel density, but is considered a good proxy (Natale et al., 2015; ICES, 2019b). Both locations with the higher prevalence of skin ulcerations (C and E; Figure 7.2) are core fishing grounds for offshore fisheries (van der Reijden et al., 2018). Previous research has also hypothesized that fishing intensity can be linked with the development of skin ulcerations (Mellergaard and Nielsen, 1997). These authors suggested the increased fishing intensity in Skagerrak region in 1989 to be associated with the increased prevalence of skin ulcerations. The hypothesis is that due to various factors during the fishing process, fish can be injured (Lüdemann, 1993; Davis and Ottmar, 2002). When they are discarded, the obtained injuries might develop into ulcerations. The link with fishing intensity might also explain the effect of the length of the fish. Since fish in the length range between 18 and 23 cm might be able to escape the net of commercial trawls or will be discarded (Seafish, 2010; Miller and Verkempynck, 2016). Both can result in abrasion of the skin which can in further stages lead to skin ulceration development. The necessity of skin abrasion for the development of skin ulcerations has already been proven in experimental trials (Vercauteren et al., 2019). However, one location, location A (Figure 7.2), has a high density of fishing vessels in spring but does not show increased skin ulceration prevalence, therefore indicating that fishing intensity is only part of the cause. This sampling site is located within the four nautical miles zone and will therefore only be visited by shrimp trawlers; possibly pointing out that gear type might also influence lesion development. To further corroborate this reasoning, more research will be necessary. Nevertheless, this study is the first to find a significant correlation between fishing intensity and lesion development demonstrating that fishing has its part in the multifactorial etiology of skin ulcerations.

Since this is only an observational study, it does not permit inferences on causality of any of the pinpointed risk-factors. Furthermore, data needs to be interpreted with care due to the low number of fish in some samplings; the limit of 50 fish, as described by Bucke et al. (1996) was not always attained. Laboratory experiments will be necessary to elucidate the implications of changing environmental factors (including climate change) on the development of skin ulcerations (Mellergaard and Nielsen, 1995).

In conclusion, this research confirmed the multifactorial aetiology of skin ulcerations in common dab in the Belgian part of the North Sea. It was evident that the increase in skin ulceration prevalence of the period 2011 – 2014 was no longer present and various fish-related, temporal and spatial factors seem to play a role in the susceptibility of common dab for the development of skin ulcerations. Furthermore, our results indicate that human activities such as fishing and climate change (with a change in sea water temperature and pH) can affect the prevalence of skin ulcerations, hereby endorsing the effect we humans have on the marine ecosystem.

7.6 Acknowledgements

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***Table 7.2**: Overview of the result of the linear regression model, with the estimate and standard error (Std.Error), the estimated z value and p-value.*

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-21.82142	6.61052	-3.301	0.000963 ***
Length	0.22978	0.05242	4.383	1.17e-05 ***
Condition	-3.39679	0.76205	-4.457	8.29e-06 ***
Temperature	0.14779	0.04228	3.496	0.000473 ***
Fishing intensity	0.12587	0.05286	2.381	0.017266 *
pH	1.73544	0.80365	2.159	0.030815 *

Chapter 8

Two-chamber skin explant model

8.1 Abstract

As in other vertebrates, fish skin consists of an outer epidermal and inner dermal layer, separated by a basement membrane. Besides sensory and osmotic functions, the skin is an important barrier between the fish and its environment. Although various cell cultures exist using scales, skin or fins, such models have limitations that complicate the extrapolation of the observed effects to the *in vivo* skin. Furthermore, to study the effects of environmental factors and/or pathogens on the skin of marine fish, the model must reckon the difference in salinity between outer and inner environment of the fish, which is, to our opinion, not feasible using a cell culture. The present study aimed to develop a reliable two-chamber skin explant model, specifically designed for marine fish. After 24 hours, the skin explant used in the model confirmed to be comparable with control skin, based on minimal histological changes in structure, number of cell layers, frequency of goblet cells and eosinophilic granulocytes and proliferative and apoptotic rates of epidermal tissue. Only a small increase of epidermal thickness in the skin explants compared to the control samples was found which was unrelated to the number of cell layers. In conclusion, the two-chamber skin explant model reported in the present study is a promising tool providing a valuable alternative for *in vivo* experiments using skin of marine fish.

8.2 Introduction

As in other vertebrates, the skin of fish consists of an outer epidermal and an inner dermal layer, separated by a basement membrane (Rakers *et al.*, 2010; Elliott, 2011a). In contrast to terrestrial vertebrates, fish epidermis mostly does not contain a dead keratinized layer. Instead, it is covered by mucus produced by goblet cells located in the upper layer of the epidermis. The mucus contains various immunologic substances such as lysozymes, immunoglobulins and lectins (Rakers *et al.*, 2010). It acts as an innate immune barrier against pathogen entry (Raj *et al.*, 2011). The skin itself exerts various essential properties including sensory functions and maintaining osmotic homeostasis, especially in marine fish (Rakers *et al.*, 2010). Moreover, it acts as a physical, chemical and immunological barrier between the fish and its aquatic habitat, an environment containing considerably more pathogens and harmful chemical substances then the aerial alternative (Noga 2000; Rakers *et al.*, 2010). Hence, skin integrity is of pivotal importance for fish health and survival (Bernet *et al.*, 1999; Ellis, 2001; Rakers *et al.*, 2010) and disruption might, amongst others, increase the susceptibility for invasion of microorganisms (Plumb & Hanson, 2011) or the uptake of toxic substances (Noga, 2000). Together, these observations substantiate the need for proper tools to study these complex interactions.

The hitherto developed *in vitro* fish cell cultures are originating from scales (Matsumoto and Sugimoto, 2007), skin (Nolan *et al.*, 2002; Lyng *et al.*, 2004; Rakers *et al.*, 2010) and fin (Chenais *et al.*, 2015) and are progressively employed in different studies using various fish species. Although cell cultures offer a relatively simple tool to study certain research questions, disadvantages are the lack of polarity, alterations of surface proteins due to digestion procedures (Pärt & Bergström, 1995) and functional changes of different cell types in culture (Nolan *et al.*, 2002). These disadvantages result in complicated extrapolation of the observed *in vitro* effects. Furthermore, *in vitro* cell cultures do not allow studying more complex interactions with the environment e.g. taking into account the difference in salinity between the outer and inner environment of marine fish.

Considering the above, the aim of the present study was to develop a two-chamber model using a skin explant including epidermal, dermal and muscular tissue. The model is specifically designed for marine fish by reckoning with the difference in salinity between the inner and outer environment.

8.3 Materials and methods

8.3.1 Animals and housing

With the Research Vessel Simon Stevin, common dab (*Limanda limanda*) were caught using a 3 m beam trawl. Trawls were limited in time to reduce the injury and stress of the fish. All macroscopically healthy fish (> 17 cm) were placed in a large survival tank, provided with oxygen and continuous renewal of the seawater. Once in the harbor, fish were transported to the Marine Station of Ostend (Flanders Marine Institute) where they were kept in recirculating tanks provided with natural seawater (16 ± 1 °C). Following seven days of acclimatization, the fish were transported to the Faculty of Veterinary Medicine (Ghent University) where they were housed in 450 L - holding tanks in groups of maximum 11 fish. The water was filtered and provided with oxygen. Water quality was monitored daily and kept in preset ranges (16 ± 1 °C; 32 ± 2 PSU; pH 8 ± 1). When the parameters exceeded these ranges, the water was refreshed and artificial seawater (Instant ocean, Aquarium systems, France) was added upon refreshment. In first instance, fish were fed with whiting

(*Merlangius merlangus*) and later on, commercially available food (Efico Sigma 874 4.5, Biomar SAS, France) was used. The use of wild-caught common dab was approved by the authorities of the Federal Government (2017/N05).

8.3.2 Two-chamber skin explant model

Design of the apparatus

The apparatus was 3D-printed (Ultimaker 2), using polylactic acid (PLA) filament. The apparatus consisted of two plates, a lower and upper plate with funnel, held together by small screws (Figure 8.1). In each plate, an opening with diameter of 18 mm was present. In both plates, ridges were provided around the opening to ensure clamping of the explant and avoid leakage. Each apparatus accommodated one skin explant. To ensure sterility of the apparatus, low temperature hydrogen peroxide plasma sterilization (STERRAD 100S) was applied.

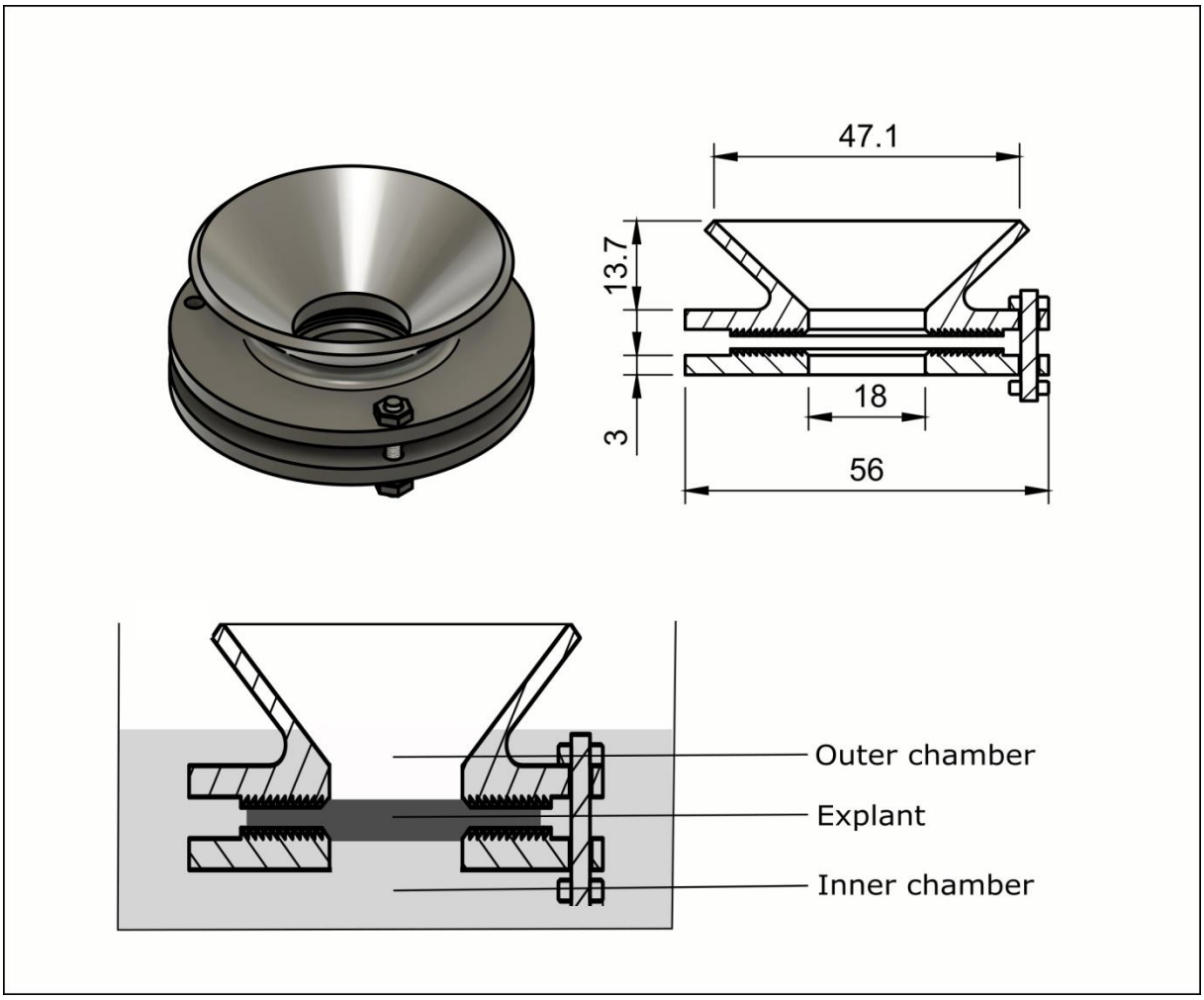

Figure 8.1 Schematic representation of the apparatus used as two-chamber skin explant model. Measures are depicted as millimeters.

Skin explant collection and disinfection

Three fish were euthanized using a stock solution of benzocaine (100 g benzocaine in 1 L ethanol) with a final concentration of 200 mg L^{-1} of seawater. Subsequently, the gill arches on both sides of the fish were cut to confirm death. Skin samples of minimally 2.5 cm by 2.5 cm and approximately 0.5 cm thick (by visual estimation) were excised using a surgical blade. Per fish, four skin explants

(EXPL) from pigmented (P) and non-pigmented (NP) side, ventral and dorsal of the lateral line were collected. Additionally, four skin samples, both from P and NP sides, served as control samples (CONT) and were immediately fixed in a phosphate-buffered 4% formaldehyde solution for histological examination. The EXPL samples were decontaminated, according to Decostere *et al.* (1999), in a bath of Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich, Belgium) supplemented with enrofloxacin (TCI, Australia) for 15 min (10 mg L^{-1}, 16°C). Thereafter, they were rinsed with sterile phosphate-buffered saline (PBS) and placed in a bath of DMEM supplemented with Polymyxin B sulfate salt (Sigma Aldrich, Belgium) for 15 min (2000 IU mL^{-1}, 16 °C).

Mounting and maintenance of the explants

Following a second rinsing in sterile PBS, the skin explants were mounted between the upper and lower plate of the apparatus. The screws were fastened to ensure proper closure and prevent leakage. Time between decontamination and mounting was kept as short as possible (< two minutes per explant). When the skin explant is mounted in the apparatus, two chambers are created, named the outer and inner chamber (Figure 8.1). The apparatus with the mounted skin explant was ultimately stored in small recipients (7 cm x 3.7 cm, 0.142 L). For maintenance of the explants, DMEM medium supplemented with 10 % fetal calf serum (FCS, HyClone, USA) and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Merck KGaA, Germany) buffer (25 mL, pH 7.0 ± 0.5) was added to the inner chamber. In the outer chamber, 3 mL DMEM medium supplemented with 10 % FCS, 10 mM HEPES buffer and sea salt (31 ± 1 PSU; Instant Ocean, Aquarium Systems, France) (pH 7.0 ± 0.5) was added. The recipients were placed on a rocking platform (150 – 200 rpm) in a temperature-controlled environment (16 ± 1°C). Both inner and outer medium were replaced after 1, 2, 3, 8, 12 and 19 hours. At these time points the explants were grossly inspected. Twenty-four hours after mounting, the explants were removed from the apparatus and fixed for minimally 24 h in a phosphate-buffered 4% formaldehyde solution.

8.3.3 Assessment of the explants

Formalin fixed tissues (CONT and EXPL) were decalcified (Decalcifying solution J.T. Baker, Avantor Performance Materials, USA), processed according to standard techniques, sectioned (5 µm) and stained, using Haematoxylin and Eosin (H&E), Periodic Acid Shiff (PAS) and immunohistochemical staining's, allowing general histological examination and assessment of skin structure and intactness.

Of each staining, five image panels were randomly acquired (x 40) along the explant cross section with Leica DMRB light or fluorescence microscope (Leica Microsystems, Belgium). Image panels were analysed as described below using scientific image software Image J (version 1.4). To avoid subjective bias during the assessment, images origins were blinded from the observers.

The thickness and number of cell layers of the epidermis were determined in five random zones in each image panel. The frequency of eosinophilic granulocytes (EGC) and goblet cells (GC) in the epidermis were assessed by counting the cells along the epidermal tissue of each panel based on the H&E and PAS staining, respectively. The frequency was expressed as number of EGC or GC per 100 µm epidermis.

To estimate the proliferation rate of the epidermis, staining of the proliferating cell nuclear antigen (PCNA) was used. After deparaffinization and rehydration of the sections, heat-induced epitope retrieval was performed using citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked in Dako REAL Peroxidase Blocking Solution (Dako, USA). Subsequently, tissue sections were incubated

with diluted mouse monoclonal anti-PCNA antibody (Abcam, UK; Ab29; mouse monoclonal antibody; 1: 10000) for 30 minutes. Visualization was obtained with anti-mouse Envision^{TM} kit and 3, 39-diaminobenzidine (DAB)+ substrate buffer. Sections were counterstained with haematoxylin followed by dehydration and mounting. Proliferative rate in the epidermal tissue was determined by counting the percentage of positive cells in a total of 100 cells assessed per image.

To detect and quantify apoptotic cell death at single cell level, terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) was performed. Non-decalcified sections were deparaffinised manually and incubated with a Sudan Black B solution, using the protocol as described by Erben *et al.* (2016). The Sudan Black solution contributes to reduce auto fluorescence and enhances the signal-to-noise ratio. Skin sections were treated with proteinase K (Dako, USA) for 25 minutes at 37°C. Apoptotic cells were labelled using the *in situ* cell death kit, fluorescein (Roche, Belgium), according to the manufacturer's instructions. Cell nuclei were counterstained with 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI; Merck, Belgium). Positive and negative controls were implemented as recommended by the manufacturer. Sections were evaluated by fluorescence microscopy. The apoptotic rate was determined by counting the percentage of positive cells in a total of 100 epidermal cells assessed in five images.

8.3.4 Data analysis

Control samples of the three fish were compared using a Kruskal-Wallis test followed by a pairwise Wilcox test to perform pairwise comparisons between group levels with the Benjamini & Hochberg (BH) correction for multiple testing. Comparisons between sides (P versus NP) and between location (dorsal versus ventral) were conducted using a two-sided Wilcox test. For these statistical analyses, R version 3.6.1 was used.

All parameters in EXPL samples were subjected to a comparative analysis in a descriptive manner (mean ± sd), with a comparison between fish and sides.

Normality of the measurements of all parameters was checked and a logarithmic transformation was performed for the proliferative rate to obtain normality. Confidence intervals (CI) for the mean were calculated per fish (replicate) and per side to estimate the difference between CONT and EXPL samples for thickness of the epidermis, EGC, GC and proliferative rate. For the proliferative rate, a back transformation was performed on the CI presented in this study for ease of interpretation. A CI for the number of cell layers, being a discrete variable, was not computed. This was therefore only analysed in a descriptive manner. Detailed CI's are available in Table 8.1.

A ratio, henceforth referred to as EXPL/CONT ratio, was calculated of each parameter by dividing the value of the parameter in the EXPL by the associated CONT sample derived from the same fish from the same side (pigmented/non-pigmented). A ratio that equals one means that EXPL and CONT samples are similar; a deviation from one indicates a difference between EXPL and CONT samples. This is a broad generalized measure but can help estimating the expected difference between EXPL and CONT samples.

Since the apoptotic rate in most of the CONT and EXPL samples was found to be zero, complicating the calculation of the confidence interval, the potential difference in apoptotic rate between EXPL and CONT samples was only analysed in a descriptive manner.

8.4 Results

8.4.1 Control samples (CONT)

All collected samples showed intact epidermal tissue and normal structural coherence. Mild exfoliation was observed in four of the control samples. Thickness of the epidermal tissue was on average 44.61 ± 8.09 µm with a maximal observed thickness of 67.56 µm. The epidermal layer consisted of 5.93 ± 0.72 cell layers. The observed EGC and GC frequency were 3.31 ± 1.59 and 4.10 ± 1.69, respectively. The proliferative rate was on average 18.96 ± 6.90 % with minimal and maximal observed rates of 8.77 % and 26.25 %, respectively. Of the analyzed CONT samples, 83 % contained no apoptotic cells and in total 95 % of the samples had apoptotic rates lower than 3 %, resulting in an average apoptotic rate of 0.42 ± 1.33 %. The maximal observed apoptotic rate was 6.12 %, although this was observed to be a localized apoptotic zone.

All samples of the three fish had similar proliferative rates ($p = 0.1784$). However, other parameters showed significant differences between fish. The main discrepancy was observed between the one fish and the other two, with significant differences in the number of cell layers ($p < 0.001$), EGC frequency ($p < 0.001$) and GC frequency ($p = 0.0030$). The thickness of the epidermal layer of control samples was significantly different in all three fish ($p < 0.001$).

Between the P and NP sides within each fish, no differences were found in the thickness of the epidermis ($p = 0.7989$) and the number of epidermal cell layers ($p = 0.5632$). Other parameters tended to be significantly different, with the main differences found in the GC frequency and the proliferative rate.

Explants collected at the same side (dorsal and ventral) were similar and none of the parameters showed consistent differences between dorsal and ventral explants.

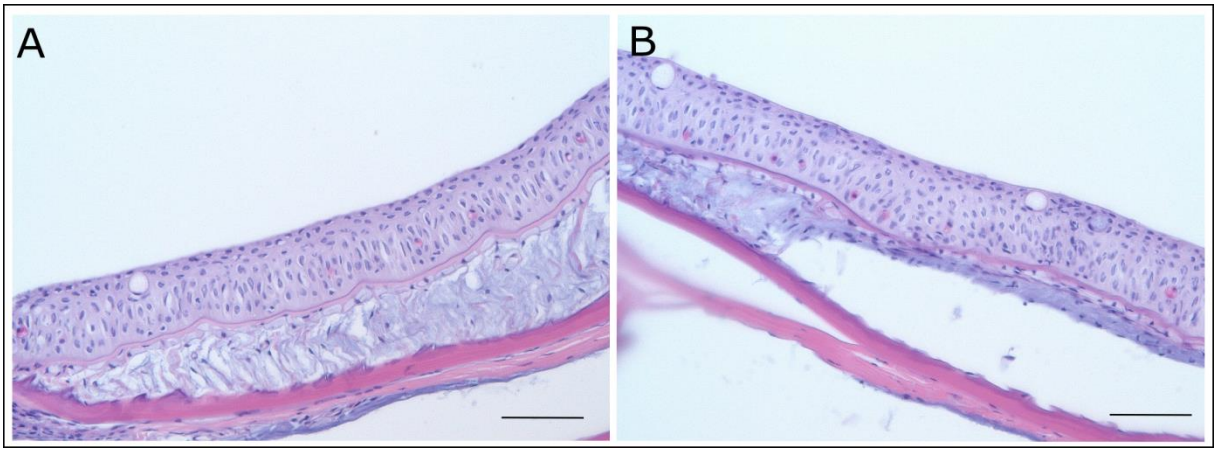

Figure 8.2: Histological image of epidermis and dermis of a control (A) and explant (B) sample demonstrating a similar morphology. The skin is intact and shows structural coherence. Scale bar = 50 µm.

8.4.2 Skin explants (EXPL)

Macroscopic examination of the explants of the P side revealed a slightly paler appearance from eight hours onwards. Upon histological examination, all skin layers described in normal skin were present in EXPL samples with normal coherence and layered structure as well as the presence of various cell types such as GC and EGC (Figure 8.2). In three explants, infiltrates of mononuclear cells

were observed mainly in the basal layer of the epidermis. Mild exfoliation was observed in seven out of 12 EXPL samples (Figure 8.3A). Furthermore, in one explant, the upper layers of the epidermal tissue were disrupted, showing reduced structural coherence and focal apoptotic zones (Figure 8.3B).

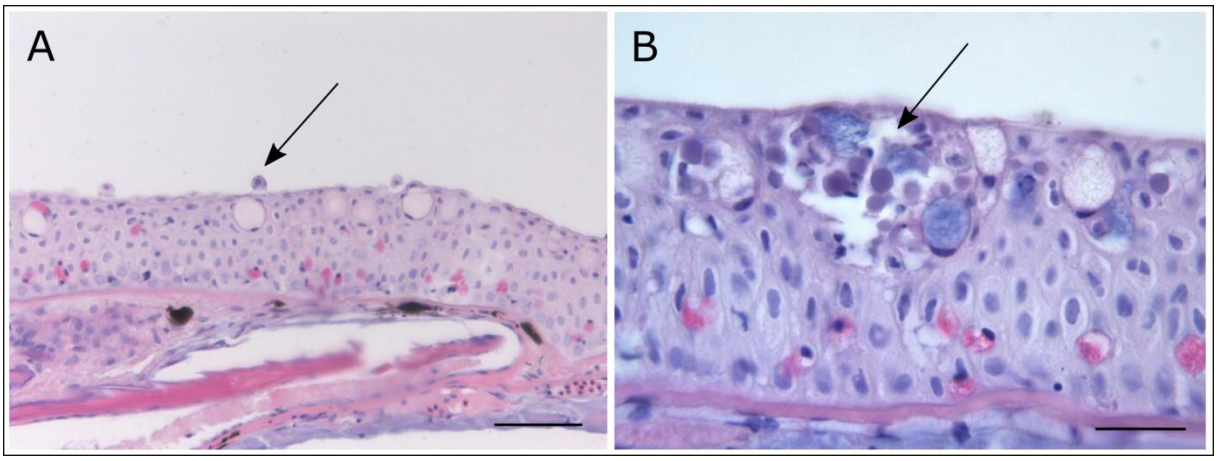

Figure 8.3: Histological details of processes observed in the skin explants. (A) Mild exfoliation was observed in both control and skin explant samples. Scale bar = 50 µm. (B) In one explant, some focal apoptotic zones were observed. Scale bar = 20 µm.

The dermis demonstrated a normal appearance, with presence of the stratum spongiosum and compactum. Melanophores of the pigmented skin in EXPL samples showed an aggregation of melanosomes (Figure 8.4). Alterations in scales were not observed in EXPL samples. In three EXPL samples, mild degeneration of the connective tissue of the stratum compactum was observed.

Degenerative changes in the muscle fibers underlying the skin were not observed, except in the peripheral part, the part between the plates of the apparatus, showing hyaline degeneration. This observation was supported by the TUNEL staining, showing apoptosis in these regions.

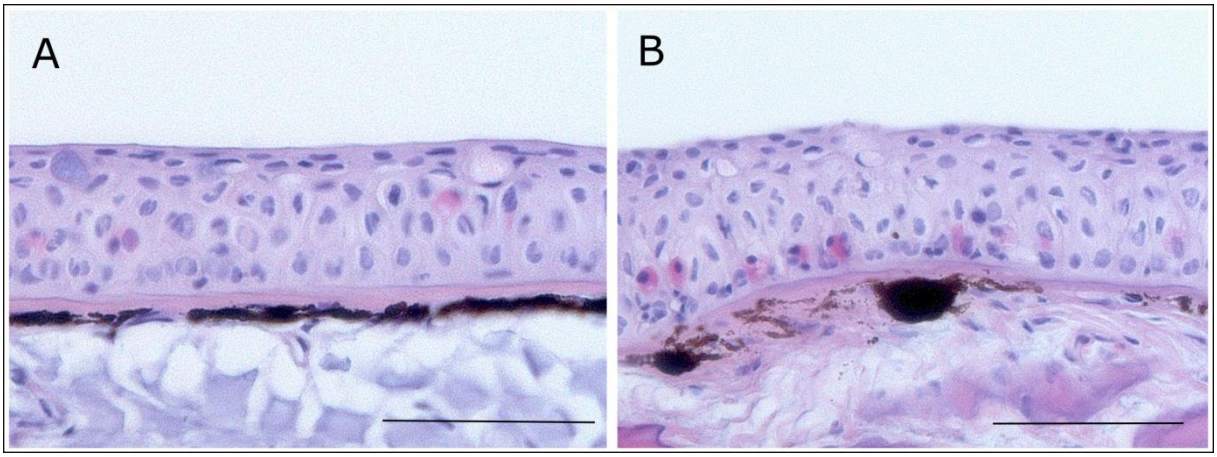

Figure 8.4: The histological difference in the melanophores in control (A) and skin explant (B) samples. Whereby in the control samples (A) a dispersion of the melanosomes is observed and in the skin explants (B) an aggregation of melanosomes, resulting in a paler (macroscopic) appearance of the skin. Scale bar = 50 µm.

The epidermal thickness of EXPL samples was on average 53.25 ± 10.54 µm, with a minimum of 29.97 µm and a maximum of 74.28 µm. The average thickness on P and NP sides were 52.77 ± 12.28 µm and 53.73 ± 9.63 µm, respectively. The number of cell layers was on average 6.23 ± 0.85, with an

average of 5.97 ± 0.96 layers on the P side and 6.50 ± 0.71 on the NP side. The maximal number of cell layers observed was eleven.

An analysis of the cell types showed that approximately 3.78 ± 1.18 EGC per 100 µm were present with similar amounts on P (3.91 ± 1.28 EGC per 100 µm) and NP (3.66 ± 1.19 EGC per 100 µm) sides. The number of EGC was variable, ranging from 1.13 up to 8.19 cells per 100 µm, although, variations seemed to be localized. The GC frequency was comparable between P (3.71 ± 0.90 GC per 100 µm) and NP (3.31 ± 1.38 GC per 100 µm), with an average GC frequency of 3.51 ± 1.13 cells per 100 µm. Again some variation was present not only between fish but also between measurements of the same explant.

The average proliferative rate was 13.55 ± 4.27 %, with 13.45 ± 5.16 % and 13.68 ± 3.52 % on the P and NP side, respectively. The proliferative rate showed variation between measurements ranging from 0.81 % to 55.81 %, with substantial variation between different measurements from one explant.

Apoptotic cells were absent in 55 % of the tissues, resulting in an average apoptotic rate of 0.92 ± 0.64 %. However, still 93 % of the tissues showed an apoptotic rate below 3 %. Maximal apoptotic rate that was measured was 9.76 %, however, this was observed to be a local apoptotic zone since another zone on the same explants contained no apoptotic cells.

8.4.3 Data analysis – comparison between CONT and EXPL samples

In most cases, no overlap of the CI was observed for the epidermal thickness, indicating an increase in epidermal thickness in the EXPL samples (Figure 8.5A). However, if the individual measures were taken into account, it can be noted that the variation between measures is substantial and results in an overlap between the actual measurements of CONT and EXPL samples. On average the epidermis was 1.18 ± 0.09 times thicker in the EXPL compared to the CONT samples. Figure 8.6 shows that the number of cell layers in CONT and EXPL samples is comparable; this is reflected in the EXPL/CONT ratio that is on average 1.04 ± 0.07.

The calculated CI's for EGC frequency showed overlap between CI of CONT and EXPL samples, indicating limited difference between both (Figure 8.5B). This was confirmed by the EXPL/CONT ratio which was on average 0.99 ± 0.26. The calculated CI's for GC frequency showed overlap in most cases, with only some minor differences observed in Replicate 2 (Figure 8.5C). Due to the variation, the average EXPL/CONT ratio was higher than one (1.20 ± 0.41).

The proliferative rate was evaluated to be similar between EXPL and CONT samples based on an overlap of the CI's of CONT and EXPL samples in most cases (Figure 8.5D). This result was supported when the individual measurements were compared showing a clear overlap and wide range of variation. The EXPL/CONT ratio did show a little difference on the NP side with CONT samples showing slightly higher proliferative rates, resulting in an average EXPL/CONT ratio of 0.58 ± 0.14. In contrast, the ratio at the P side was 1.36 ± 0.40. Multifocal zones of increased and/or decreased proliferation were observed in both EXPL and CONT samples, leading to increased variation.

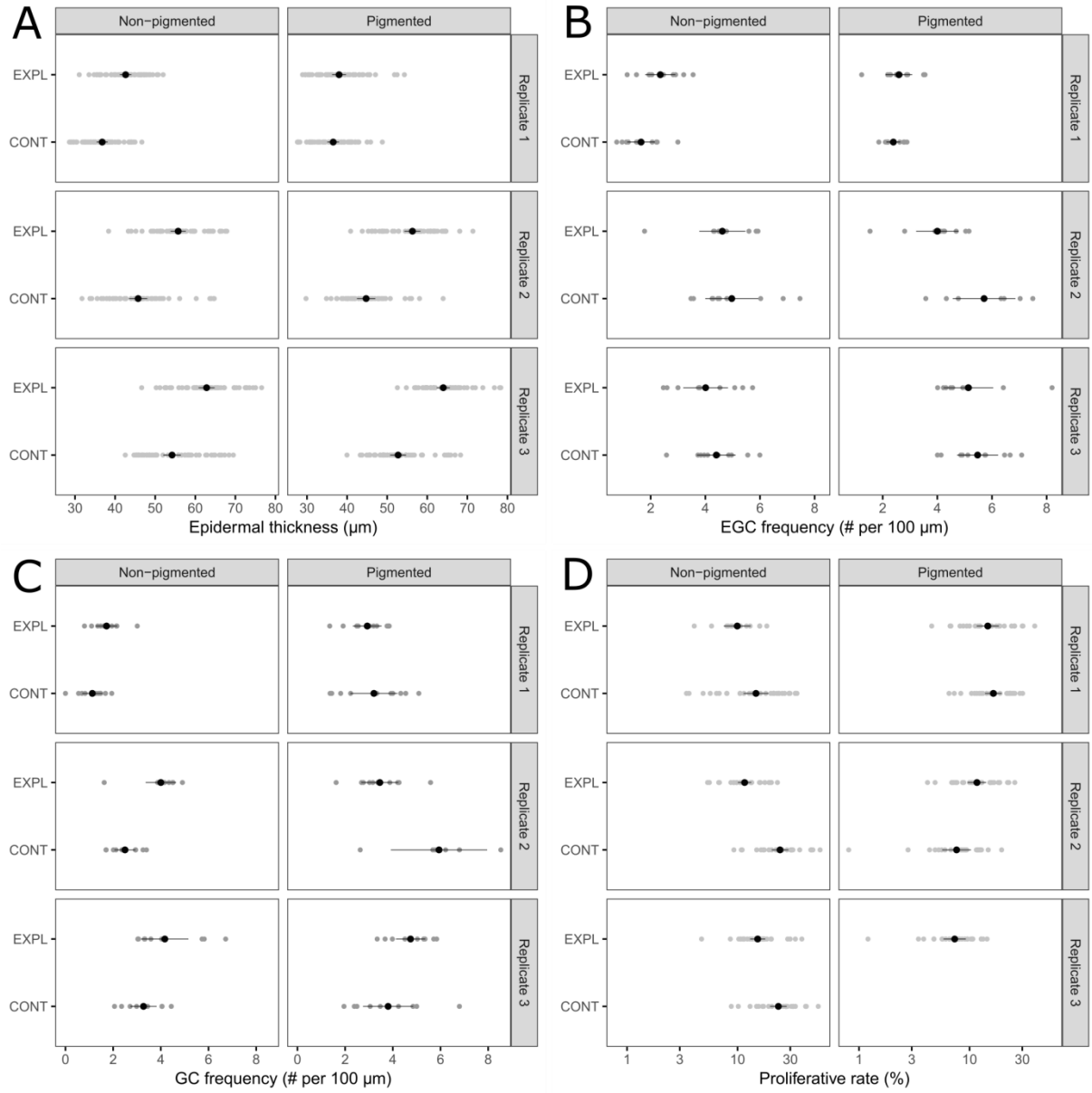

Figure 8.5: Overview of the calculated confidence intervals for the mean of (a) epidermal thickness, (b) eosinophilic granulocytes (EGC), (c) goblet cells (GC) and (d) proliferative rate. Due to the variation between sides and fish, the measures are depicted per side (Non-pigmented/pigmented) and per fish (Replicate). In black the CI is depicted and the actual measurements are depicted as grey dots. Since the proliferative rate was not normally distributed, the analysis was performed using a logarithmic transformation. For ease of interpretation, back transformed values and CI's are used.

Both CONT (95 %) and EXPL (93 %) samples showed similar percentages of tissues with apoptotic rate below 3 %, although the number of samples without any observed apoptosis was lower in EXPL samples.

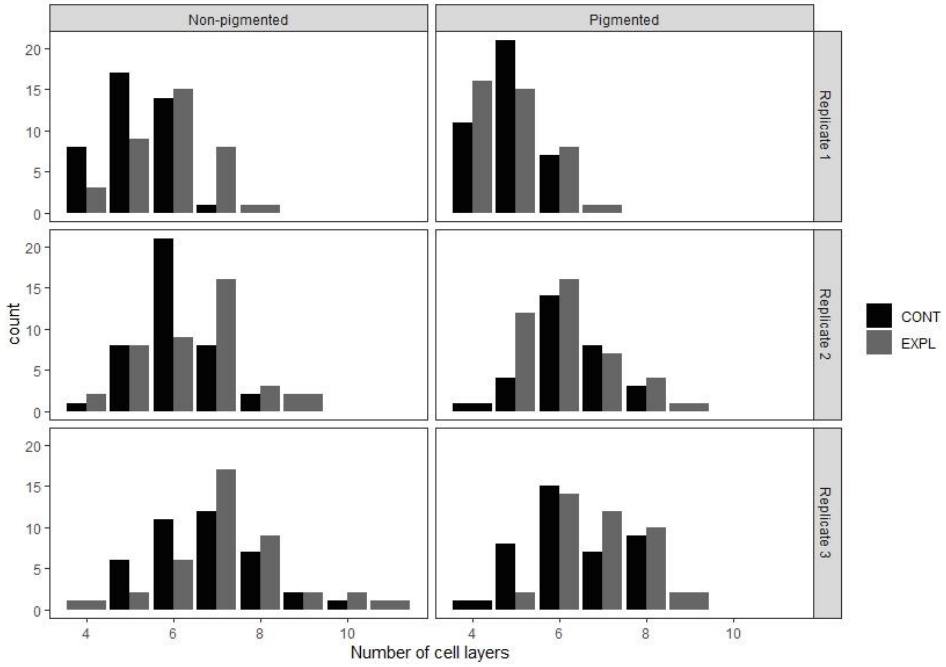

Figure 8.6: Histogram visualizing the number of cell layers that was counted, stratified per side (Non-pigmented/pigmented) and fish (Replicate). A clear overlap was observed between the number of cell layers present in CONT (Black) and EXPL (Dark gray) samples. Even within a sample, substantial variation can be present.

8.5 Discussion

In this study, an innovative and unique two-chamber skin explant model was developed enabling the study of various toxicological and pathogenic impacts on skin of marine fish. The model considers the difference in salinity between the outside and inside environment of the fish and has demonstrated to be a reliable and easy-to-use model. After 24 h, the explant skin used in the model confirmed to be comparable with normal skin of common dab, based on minimal histological changes and the absence of increased degeneration or regeneration.

This *in vitro* model contributes to the "Reduction, Refinement and Replacement" of the 3R principle of animal experimenting (Russell & Burch, 1959). When larger fish (> 17 cm) are used, up to eight explants can be collected. This allows the evaluation of multiple treatments and results in a reduction of the variability and the number of experimental animals needed without loss of statistical power. The capability of fish for nociception and experiencing pain is becoming more and more accepted (Sneddon, 2019). The use of the two-chamber skin explant model replaces *in vivo* studies in which fish are exposed to detrimental contaminants or pathogens which might cause pain or discomfort. The fish used in our model are humanly killed by an overdose of anesthetic, therefore contributing to the "Refinement" of animal experimentation (Russell & Burch, 1959). Further reduction of animal pain and distress could be accomplished by replacing the use of fetal calf serum in the medium by possible alternatives such as human platelet lysates. However, more research will be necessary to estimate the impact on the skin explant (Jochems *et al.,* 2002; van der Valk *et al.*, 2017).

Although no large differences between the skin of the explants and the control skin were observed, some changes occurred which should be considered when using the two-chamber skin explant model. Macroscopically, explants of the pigmented side showed a paler appearance. This observation was confirmed histologically and not attributed to necrosis of melanocytes but rather caused by an intracellular aggregation of melanosomes, causing tissue to appear lighter (Svensson & Sköld, 2011). *In vivo*, color change can be regulated by nervous, endocrine and paracrine stimulations (Svensson & Sköld, 2011). In skin biopsies of various fish species, similar changes were observed in presence of melatonin, noradrenalin, endothelins, melanin-concentrating hormone (MCH) and/or light (Svensson & Sköld, 2011). Although the cause of the observed pallor is unknown, its impact on the viability and reliability of the model is estimated to be low.

Although individual measurements of the epidermal thickness of control and explant samples showed a clear overlap, the confidence intervals did not. We can therefore assume that the epidermis of the skin explants was on average 1.18 times thicker compared to the control samples. Since the number of cell layers remained stable, an increase in cell size or intercellular spaces can be the cause of the observed thickening. These changes could however not be histologically confirmed. Functional studies should be performed to examine this phenomenon since cellular swelling could be attributed to changes in osmoregulation, a very important mechanism in saltwater fish.

The main proliferative and apoptotic rates of the skin explant were 13.55 % and 1.03 %, respectively, and were not different from rates observed in control samples, demonstrating the lack of cell death and/or subsequent proliferative compensation. Since the epidermis is a metabolic active tissue, proliferation and apoptosis can be expected in all layers of the epidermis (Nolan *et al.*, 2002; Elliott, 2011a). Nevertheless, differences are observed between the pigmented and non-pigmented side. This could reflect a natural phenomenon in flatfish although further research is necessary to elucidate the cause of this difference. In addition, we have observed in both explant and control samples, focal areas of increased proliferation and/or apoptosis, endorsing the importance to examine multiple ad random sites.

Various skin parameters, such as the thickness of the epidermis, are known to be prone to variation due to species, ages, location on the body and environment (Lindesjöö *et al.*, 1994; Elliott, 2011a). Also differences between the pigmented and non-pigmented sides of flatfish are reported, albeit seldom (Yamamoto *et al.*, 2011; Spinner *et al.*, 2016). In our study, variation was confirmed both between fish and within the fish and between P and NP sides. Due to the low number of fish used in this study, no correlations with fish-related characteristics can be examined. However, since various explants can be collected from one fish, a control sample from both sides of the flatfish should be included to consider this variability and various measurements need to be conducted on each sample.

This model is believed to be reliable and useful for skin studies, despite the small changes that were observed between the explants and control samples. However, with each research question, the benchmark for biological relevance can be different, based on the expected magnitude of the effect. In future studies applying this model, an equivalence test or effect size statistics (using the magnitude of the effect and the confidence interval of the results) should be performed to draw a clear conclusion based on biological relevance and statistical significance (Nagawaka & Cuthill, 2007).

In conclusion, the described two-chamber skin explant model could be a promising tool providing a valuable alternative for *in vivo* experiments using skin of marine fish. The proposed protocol has been proven to closely approximate the *in vivo* skin structure and its cellular composition. Based on the results presented here, it is believed that the developed two-chamber skin explant model for marine fish, will lend itself for many future applications including the study on the effect of toxic compounds on the total skin structure and integrity as well as disease related aspects such as host-pathogen interactions or the uptake of contaminants.

8.6 Acknowledgements

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Table 8.1: *Calculated confidence intervals (lower and upper limit) around the mean for epidermal thickness, eosinophilic granulocyte (EGC) frequency, goblet cell (GC) frequency and proliferative rate. Importantly, the depicted values of the proliferative rate were back transformed for ease of interpretation.*

			Epidermal thickness (µm)			**EGC frequency (# per 100 µm)**			**GC frequency (# per 100 µm)**			**Proliferative rate (%)**		
Time	Side	Test	Lower	Mean	Upper	Lower	Mean	Upper	Lower	Mean	Upper	Lower	Mean	Upper
CONT	Non-pigmented	Replicate 1	35.41	36.78	38.15	1.14	1.65	2.15	0.70	1.13	1.55	11.34	14.68	19.01
EXPL	Non-pigmented	Replicate 1	41.19	42.63	44.07	1.80	2.35	2.90	1.28	1.72	2.17	7.51	9.93	13.14
CONT	Pigmented	Replicate 1	35.09	36.54	37.98	2.14	2.39	2.64	2.22	3.21	4.20	13.82	16.43	19.52
EXPL	Pigmented	Replicate 1	36.28	38.00	39.73	2.11	2.59	3.08	2.32	2.93	3.53	11.60	14.62	18.41
CONT	Non-pigmented	Replicate 2	43.46	45.75	48.03	4.00	4.97	5.94	2.06	2.50	2.94	20.37	24.22	28.81
EXPL	Non-pigmented	Replicate 2	53.85	55.74	57.63	3.78	4.62	5.47	3.36	4.00	4.64	10.08	11.58	13.31
CONT	Pigmented	Replicate 2	42.47	44.75	47.03	4.56	5.71	6.86	3.92	5.94	7.96	5.65	7.63	10.31
EXPL	Pigmented	Replicate 2	54.30	56.30	58.30	3.22	4.00	4.78	2.68	3.46	4.23	9.61	11.67	14.16
CONT	Non-pigmented	Replicate 3	52.03	54.22	56.41	3.70	4.40	5.11	2.72	3.27	3.82	19.70	23.40	27.79
EXPL	Non-pigmented	Replicate 3	60.83	62.83	64.82	3.19	4.01	4.83	3.18	4.17	5.16	13.00	15.21	17.80
CONT	Pigmented	Replicate 3	50.75	52.73	54.71	4.72	5.48	6.23	2.75	3.80	4.86	5.81	7.33	9.25
EXPL	Pigmented	Replicate 3	62.38	64.00	65.63	4.23	5.14	6.05	4.13	4.74	5.36	11.34	14.68	19.01

Chapter 9

General discussion

9.1 Framework, aims and value of this study

Despite the relatively high prevalence of skin ulcerations in flatfish, species with an ecological and economical importance, the exact etiology of these lesions is still unknown. This lack of knowledge has its roots in the complexity of the fish, the marine environment that they inhabit and the intimate relation between both (Rakers *et al.*, 2010). Nonetheless, diseases are ecological important since the occurrence of diseases and changes in their prevalence can be used as a proxy for the health status of the entire ecosystem, stressing the need to fill this gap of knowledge.

A general framework for understanding this complex etiology of diseases was provided by Snieszko (1974). He proposed a model with three interdependent factors – fish, pathogen and environment – which, when in imbalance, can result in the development of an infectious disease (Chapter 1 - Figure 1.5). Due to this, an integrated, comprehensive and multidisciplinary research is necessary to increase the scientific understanding of the etiology of skin ulcerations in common dab (*Limanda limanda*) and the risk-factors involved in their development. This was the overall aim of the current PhD research using the framework as proposed by Snieszko (1974) as a guideline throughout this work. This resulted in three main parts studying the involvement of pathogenic agents, fish-related characteristics and environmental factors.

This general discussion tackles some comprehensive points in light of the combined research findings. This chapter starts with an overview of the used model species, methodologies and ethical considerations of using wild-caught common dab in experimental situations. This is followed by a summary and critical analysis of all research results regarding the etiology and risk-factors involved in the development of skin ulcerations in common dab, including pathogens, fish-related factors and environmental factors. This is mainly based on our results but integrated with current scientific knowledge. Some remarks regarding the ability to extrapolate the research results are written down. This general discussion is continued with some considerations regarding the possible impact of skin ulcerations on the health and survival of fish. Subsequently, retrospection on the sudden increase in skin ulcerations between 2011 and 2014 is offered, with an attempt to formulate some hypotheses that might explain this observation based on the knowledge gathered in this PhD research and existing literature. This is followed by a short review of some existing policies related to the monitoring of fish diseases, with policy recommendations based on the findings of this PhD study. A general conclusion is formulated integrating the impact of pathogenic agents, fish-related characteristics and environmental factors in the development of skin ulcerations in common dab. Throughout the discussion, various future research topics and perspectives will be mentioned.

9.2 Research methodology

Establishing a cause-effect relationship for infectious diseases is a nontrivial task (Vethaak & ap Rheinhallt, 1992). Before we dive into the main results of this research, we would like to start with a critical analysis of its building blocks (the fish and experimental methodologies). We believe that the combination of all experimental approaches chosen during this research are complementary, support the multifactorial etiology and guarantee extrapolation of the research results (Calisi & Bentley, 2009). Since any experimentation using living animals might elicit some questions regarding the fish welfare, we conclude this part with some ethical considerations on the chosen research methodologies.

9.2.1 Choice of fish species

As mentioned in the introduction, common dab was used as bio-indicator species in biological effect monitoring programs (Vethaak *et al.*, 2009; Lang *et al.*, 2017) and the International Council for Exploration of the Sea (ICES) fish disease monitoring. During these monitoring campaigns, the prevalence of various diseases, amongst which skin ulcerations, is studied (Bucke *et al.*, 1996). Common dab is omnipresent in the North Sea and sensitive for development of different diseases. Therefore, the choice of using this species in research studying skin ulceration development is highly supported and adds a great value to this study and its results.

To our knowledge, common dab has (not yet) been cultured nor is this species commercially available, inevitably resulting in the use of wild-caught common dab in the experiments. This unavoidably entails a lack of controllability and increased variability in the experimental population. The collected fish will have different length, life history, age, and immune status. No information will be available on the disease history of the fish which in turn can have an uncontrollable effect on the disease resistance. Furthermore, it is possible that fish have a different genetic background. For example, genetic variability in genes of the major histocompatibility complex (MHC) has proven to be linked to disease resistance (Arkush *et al.*, 2002). All these elements can result in variable susceptibility between fish to the development of skin ulcerations. Finally, wild-caught fish will not be free of pathogens, in contrast to some commercially available specific-pathogen-free (SPF) laboratory animals. On the contrary, the skin houses its own diverse microbial flora, possibly containing pathogenic microorganisms. This was experienced in both infection experiments (Chapter 4 and 6), where the bacteria under study, *Vibrio tapetis* and *Aeromonas salmonicida,* were isolated from clinically healthy fish at the beginning and control fish at the end of the experiment. Due to all these factors, the use of wild-caught fish adds some complexity to the interpretation of the scientific results, however, it also ensures representativeness and environmental relevance of the research results since the natural variability of a population is believed to be represented in the experimental population. Therefore, the choice of common dab as experimental species is substantiated.

9.2.2 Experimental methodology

To study the etiology and contributing factors to the development of skin ulcerations in common dab, three experimental approaches were employed; (1) fish disease monitoring *in situ* (at sea), (2) *in vivo* experimental trials and (3) *in vitro* experiments. Evidently, the experimental approach was chosen based on the research questions, however, every approach has its advantages and disadvantages.

***In situ* fish disease surveys** use fish in their natural environment, allowing a comprehensive study to investigate changes in skin ulceration prevalence and fish-related as well as environmental risk-factors associated with disease development (Chapter 3 and 7). These studies add valuable information and can offer new insights in complex disease etiologies. Surveys at sea are technically and practically challenging since the marine environment is highly variable in time and space and various factors can cause confounding of the results. Strong correlations between factors might also conceal the exact causal agents (Wosniok *et al.*, 2000). Moreover, linked to the numerous factors that define the marine environment, often unknown or unrecorded parameters can play a role in the disease occurrences, therefore impeding clear conclusions (Dethlefsen, 1990; Calisi & Bentley, 2009).

***In vivo* experiments** are a useful tool to investigate direct cause-effect relationships (Calisi & Bentley, 2009). The greatest advantage of such experiments is the ability to create controlled experimental conditions (temperature, salinity, controlled infection,…), resulting in 'clean' scientific results and thus facilitating their interpretation (Chapter 4 and 6). Nevertheless, the unnatural experimental environment will lack the complexity of the natural marine environment.

As a mean to study possible synergistic or additive effects, an ***in vitro* experimental method** was developed as described in Chapter 8. The innovative two-chamber skin explant model can be a starting point for various future studies, not limited to skin ulcerations but applicable for the study of various skin diseases of marine fish. The use of a small skin sample minimizes or eliminates the effects of confounding factors. By using multiple explants and thus multiple treatments per fish the impact of variability between fish can be greatly reduced. Nevertheless, inherently linked to the distance to a natural situation, the extrapolation of the results gathered is very complicated. The use of the *in vitro* model might also elicit some critical remarks. The explant model is perfectly adapted to use in small scale experiments, but it will be less appropriate on larger scale due to the labor intensive experimental design. Moreover, a good condition and reliable results of the explant can, for now, only be guaranteed for 24 h. Although this will suffice to study for example the initial adherence and invasion of pathogens in the host tissue, it will not be possible to study later stages of skin ulceration development. More experimentation and possibly changes in the protocol will be necessary to explore the possibilities to keep the skin explants for a longer time period.

Complementary aspects of the research approaches

While *in situ* field experiments have high environmental relevance, they often have an observational character and thus do not allow pinpointing cause-effect relationships. In contrast, laboratory experiments have great potential to identify causal links between factors. However, its environmental relevance can often be questioned due to the deviation from the natural environment and complexity (Vignati *et al.*, 2007; Hernán, 2018). Although field validation of laboratory results is always necessary, it is not straightforward. During this research, we tried to reduce the gap between the laboratory and the field as much as possible by using wild-caught fish, considering ecological traits and life histories of organisms and using field-based experimental conditions (Vignati *et al.*, 2007; Forbes *et al.*, 2008; Calisi & Bentley, 2009). By these measures we try to increase the natural variability and uncontrollability, pursuing the representativeness of the experimental research results as much as possible. Furthermore, by the combination of field and laboratory experiments, this extrapolation was also simplified. Nonetheless, generalization from experimental research to the field must still be done with great caution.

9.2.3 Ethical considerations

The choice of research approaches is inherently linked to questions regarding the ethical impact of the experiments on the fish. The capability of nociception and pain (emotional component) in fish is generally agreed upon and supported by scientific anatomical evidence; however some sceptics still deny this (Braithwaite & Boulcott, 2007; Sneddon, 2009; Diggles *et al.*, 2011; Sneddon *et al.*, 2018; Sneddon, 2019). The ability of fish to feel pain and discomfort highlights the importance of taking fish welfare into consideration when performing *in vivo* experiments. Furthermore, the experience of discomfort mostly results in physiological and/or behavioral changes, which in their turn can affect scientific results or outcomes of *in vivo* experiments, emphasizing the importance of taking fish welfare into account in experimental settings.

Nevertheless, in order to consider possible effects on animal welfare, knowledge on how to recognize symptoms predicting stress, anxiety and death is of pivotal importance. In 2018, in total 30921 fish were used in experimental trials in Flanders of which 16941 zebrafish (*Danius rerio*) and species such as common carp (*Cyprinus carpio*), burbot (*Lota lota*), rainbow trout (*Oncorhynchus mykiss*), thornback ray (*Raja clavata*) and goldfish (*Carassius auratus*) (Departement Omgeving, 2019). The limited knowledge for not commonly used species such as common dab and the existing natural variation make it very challenging to recognize the symptoms predicting anxiety, stress and death. Increased knowledge would help us to recognize these signals, improve welfare and formulate valuable humane endpoints (defined as "a refinement strategy designed to minimize pain, suffering or distress experienced by animals during an experiment") (Hendriksen *et al.*, 2011).

During various *in vivo* and *in vitro* experimental studies, we build up some experience on symptoms of decreased welfare in common dab. The best option to estimate welfare of the common dab appeared to be visual observation of behavior and feeding response whereby aberrant behavior (no reaction upon catch or reduced movement over various days) and loss of feeding response (for multiple days) appeared to be linked with mortality, although it is not a flawless method. Other, more quantitative measures were evaluated such as the use of the "reflex action mortality predictors (RAMP)" method (Davis & Ottmar, 2006; Davis, 2005; Davis, 2010) and detailed observation of behavior using video recordings. The first method is based on the assessment of six easily tested reflexes whereby the impairment of one or more of these reflexes might predict mortality. This method is mainly used to estimate the vitality after catch and estimate bycatch survival using field-based, laboratory holdings or telemetric analysis (Davis, 2007; Raby *et al.*, 2011; Uhlmann *et al.*, 2016). However, in an experimental situation, regular assessment of reflexes resulted in increased stress and handling, which in its turn could induce the development of lesions or mortality. Furthermore, some fish without impairment of reflexes died the day after RAMP observations, rendering decreased prediction capacity in common dab in this context. Observing and interpreting behavioral changes is complicated since no information is available on 'normal' or 'natural' behavior of common dab, let alone expected behavior in experimental situations.

Our knowledge regarding symptoms predicting stress, anxiety and death were based on experiences; this clearly emphasizes the urge for more research and the need for a framework for evaluating wild-caught fish welfare during experimental situations.

9.3 Research results: unravelling the etiology

In the following section, the main results will be summarized and discussed in view of the combined findings of different studies, again subdivided in results related to pathogenic agents, fish-related characteristics and environmental factors.

9.3.1 Pathogenic agents

Vibrio tapetis* and *Aeromonas salmonicida

The pathogenic agents, *Vibrio tapetis* and *Aeromonas salmonicida*, that were isolated in active ulcerations during the explorative study (Chapter 3), are recurring in several studies performed during this study. Both *V. tapetis* and *A. salmonicida* were found to play a role in the development of skin ulcerations based on the *in vivo* immersion challenges (Chapter 4 and 6). The involvement of *V. tapetis* was further confirmed based on the data gathered during the four-year survey where *V.*

tapetis was isolated in 23 % of the lesions. It was even found in pure culture in one fish, stressing its clinical relevance (Chapter 7).

Vibrio tapetis is a well-known pathogen causing Brown Ring Disease (BRD) in bivalves with Manila clam (*Ruditapes philippinarum*), an economically important aquaculture species, as the most susceptible species (Paillard *et al.*, 1994; Paillard, 2004b; Paillard, 2017). Since 2003, first reports of *V. tapetis* in fish causing various clinical signs ranging from loss of appetite to development of lesions and death were published (Jensen *et al.*, 2003; Reid *et al.* 2003; López *et al.*, 2011; Declercq *et al.*, 2015). The isolation of various isolates in fish, including in common dab, had raised some questions on their host-specificity and possible virulence markers involved. Although we were not able to pinpoint any host-related differences, based on the protein profiles, we could discriminate between pathogenic and non-pathogenic isolates for clam species (Chapter 5). This indicates a difference between both *V. tapetis* types, raising even more questions on the phylogeny of this bacterium.

In the explorative study, *A. salmonicida* was also isolated from wild fish, one time as a pure culture and the other in co-culture with *V. tapetis* and/or other bacterial species (Chapter 3). In the subsequent four-year survey (Chapter 7), *Aeromonas sp.* was isolated in one ulceration, although the identification was not reliable on species level. The *in vivo* challenge trial indicated that *A. salmonicida* may indeed be involved in the development of skin ulcerations, although its relevance needs to be investigated further (Chapter 6). An explanation for this somewhat more complicated result is the difficulty to re-isolate *A. salmonicida* from ulcerations (Austin & Austin, 2012). This was already reported and can be linked to slow-growing phenotypes resulting in uncompetitiveness with faster growing, opportunistic bacteria (Austin & Austin, 2012). Nonetheless, the detailed study on the A-layer protein, a well-known virulence factor, showed the presence of a previously undescribed A-layer type (Chapter 3; Gulla *et al.*, 2019). The A-layer, encoded by the virulence array protein (*vapA*) gene, forms an additional external layer on the cell wall which protects the bacterium during invasion in the host tissue (Garduño & Kay, 1992; Gulla *et al.*, 2016; Hamed *et al.*, 2018). It is clear that the isolates that were retrieved from skin ulcerations in common dab have a specific A-layer type that does not cluster with *vapA* sequences of previously known *A. salmonicida* strains and was, so far, not isolated in other species than dab (Figure 9.1) (Gulla *et al.*, 2019). This distinct clustering of A-layer types presumably according to host-species might be regarded as evidence that *A. salmonicida* (A-layer type 15) is indeed adapted to protect itself during colonization of the skin of common dab specifically.

Although both bacteria might play a role in the development of skin ulcerations, their mode of action might be different. In the experiment using *V. tapetis*, high mortality rates occurred only four days after infection. Furthermore, *V. tapetis* was mainly isolated in the lesions of fish that died and the bacteria was never found in lesions sampled at the end of the experiment (Chapter 4). In contrast, fish that were infected with *A. salmonicida*, mortality was observed starting 10 days post infection and differences in severity of lesions were mainly found in that same period (Chapter 6). Furthermore, *A. salmonicida* was isolated more frequently from lesions at the end of the experiment. Based on these results, it appears as if *A. salmonicida* has a longer incubation time compared to *V. tapetis.* However, one could argue that this is substantiated by the reported preference of *A. salmonicida* for adherence to and destruction of muscular tissue (Ellis *et al.*, 1981). The longer incubation time was already reported (Wiklund, 1995) although the opposite was also found with quite short incubation times of *A. salmonicida* in Atlantic salmon (*Salmo salar*) (Svendsen & Bøgwald,

1997). The use of culture-independent, molecular methods, as employed by Goodwin and Merry (2009) might be necessary to gain a good perspective on the role of *A. salmonicida* in the development of skin ulcerations (Toranzo *et al.*, 2005).

Although the research results are inconclusive on the underlying pathogenicity, it is tempting to speculate that prior infection with a first pathogen, such as *V. tapetis*, might facilitate the infection by *A. salmonicida*. Importantly, both experiments were performed using a different experimental population of common dab, caught at a different time. Therefore, differences in population structure, microbial history or bacterial flora present on the skin can also have an impact on the results.

Other potentially involved pathogenic agents

It is important to keep in mind that only a subset of pathogenic agents were studied in this research. This is inherently linked to the used methodology of bacterial examination. Bacterial samples of skin ulcerations were plated on bacterial agar to induce growth, allow purification and subsequent identification. However, not all marine bacteria are cultivable on agar plates, therefore it is possible that important bacterial species are not recorded but might play a role in ulceration development. Furthermore, slow-growing bacteria can be outcompeted by faster-growing species. DNA-based methods would be more suitable to identify all bacteria involved in lesion development (Goodwin & Merry, 2009).

Furthermore, during the monitoring survey, other *Vibrio* species such as *V. alginolyticus* and *V. tasmaniensis* were also repeatedly isolated in active skin ulcerations (Chapter 7). Therefore, might also be able to cause skin ulcerations in common dab, although experiments should be performed to ascertain a causal inference.

Outside-in and inside-out

During this research we focused on the “outside-in” theory of skin ulceration development (Law, 2001), defined as an ulcer that started from the outside. However, during the experimental study described in Chapter 4, we discovered multifocal, raised irregular lesions with a diameter between 0.2 and 1 cm on the skin of 12 % of the fish in the experiment. Further histological examination of these lesions revealed inflammatory infiltration in the *stratum spongiosum* and *compactum* and presence of multinucleated osteoclasts in the scale pockets (Vercauteren *et al.*, 2019b). Some of these lesions also showed ulceration of the skin, starting from below the skin surface and working its way outwards, substantiating the “inside-out” hypothesis (Law, 2001). Based on the epidemiology, clinical signs and histopathological features, similarities were found with the so called Red Mark Syndrome (RMS) described in rainbow trout (McCarthy *et al.*, 2013; Oidtmann *et al.*, 2013). Although we were not able to pinpoint the cause of these lesions, *Rickettsia*-like bacteria (Alphaproteobacteria) or bacteria of the family *Midichloriaceae* (Alphaproteobacteria) are suspected to play a role as pathogenic agents in this pathology in rainbow trout (Metselaar *et al.*, 2010). This stresses again the multifactorial causality of skin ulcerations in common dab.

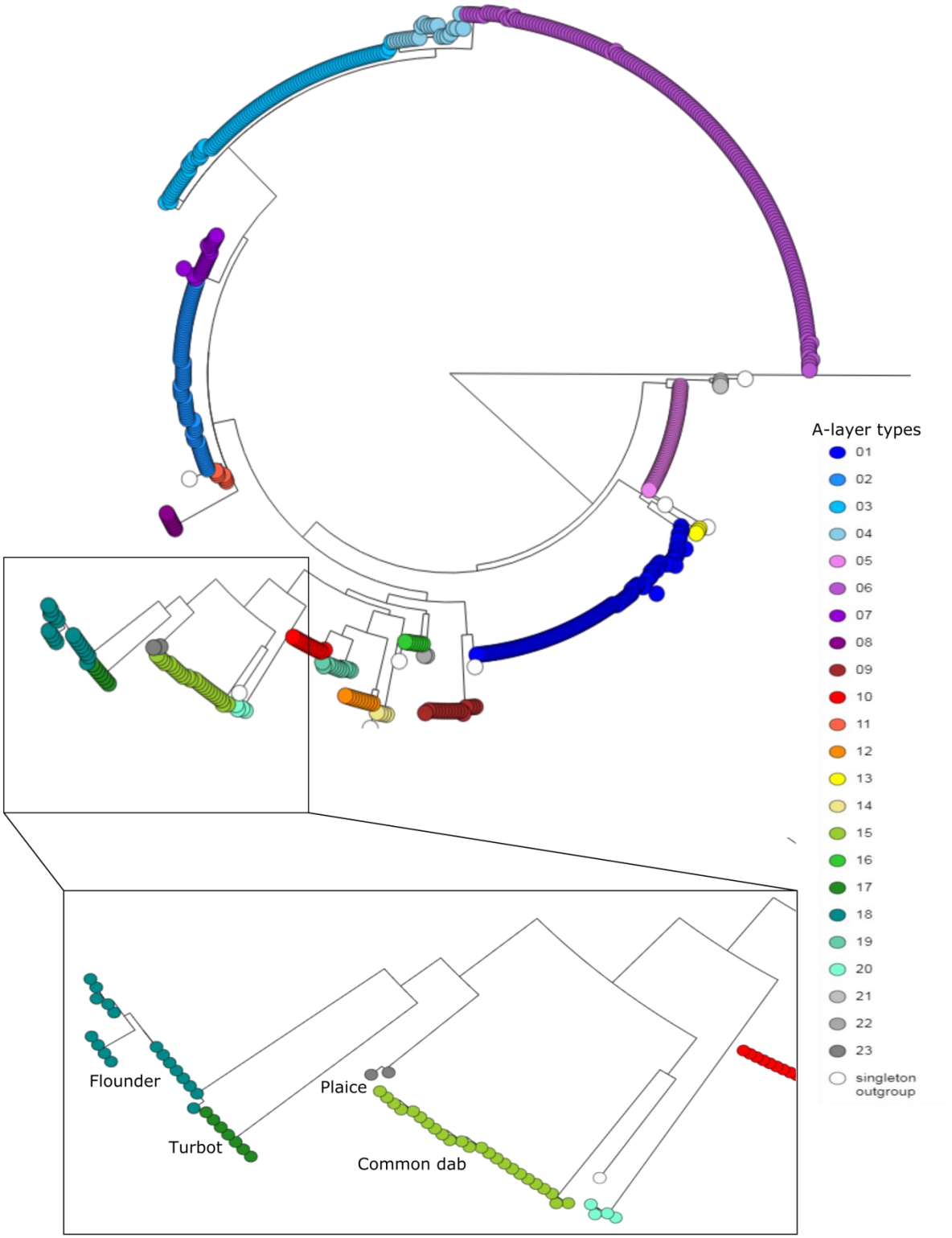


Figure 9.1: Dendrogram based on the partial vapA nucleotide sequences. The clusters represent the different A-layer types (depicted by different colors). The insert shows a close-up of the A-layer type clustering of flatfish. The tree was extracted from the microreact project as proposed in Gulla et al. (2019).

9.3.2 Fish-related characteristics

Various fish-related characteristics can modify the susceptibility of the fish to develop a skin ulcer.

Pigmented and non-pigmented side

One specific characteristic of common dab, and all flatfish, is the clear difference between the pigmented or eye side, facing the water column, and the non-pigmented or blind side, facing the sediment (Spinner *et al.*, 2016). The pigmented side is equipped to perfectly adjust to the color and pattern of the sediment, playing an important role in camouflage of the fish (Burton, 2010). Although one might expect morphological or even functional differences between both sides, merely a handful of studies investigated this phenomenon. Faílde *et al.* (2014) showed that, besides a difference in pigmentation, a higher number of mucus-producing goblet cells with an occasional observation of clusters of such cells were found on the pigmented compared to the non-pigmented side in turbot (*Psetta maxima*). During this research, a small-scale study was performed although this difference could not be confirmed for common dab (Vroman et al., 2019). A difference in scale morphology was also pinpointed recently; this is believed to be related to retaining the sand (Spinner *et al.*, 2016).

The difference between the pigmented and non-pigmented side of flatfish and possible differences in susceptibility to ulcer development have always been a point of interest for many scientists, however without reaching a consensus (Vethaak *et al.*, 1992; Wiklund, 1994). Vethaak (2013) reported higher incidences of skin ulcerations on the non-pigmented side in wild-caught flounder (*Platichthys flesus*). Wiklund and Bylund (1993) reported a sex-related distribution of ulcerations on pigmented and non-pigmented side of flounder. Even in our studies, contradicting observations were found with the development of more, severe and larger ulcerations on the pigmented side during the *in vivo* experiments (Chapter 4 and 6) and higher prevalence of ulcerations on the non-pigmented side during the survey at sea (Chapter 7). The reason(s) for these apparently divergent findings hitherto remain(s) obscure. A possible explanation can be that the experimental protocol as used in chapter 4 and 6 might benefit the development of skin ulcerations on the pigmented side. Since the fish remained mainly on the bottom and the bacterial suspension was added in the tank water during inoculation, the bacterial load on the pigmented side was expected to be higher compared to the bacterial load on the non-pigmented side. The situation at sea can be expected to be different since pathogens can reside in the sediment, hereby exposing the non-pigmented side to higher bacterial concentrations. This might explain the difference observed in field and experimental situations.

Skin surface condition

The skin barrier is known to be one of the most important defence mechanisms of fish against various pathogenic micro-organisms or toxic compounds in the marine environment (Svendsen & Bøgwald, 1997). As was apparent in both *in vivo* experimental studies performed (Chapter 4 and 6), prior skin damage acts as a major contributing factor in the development of skin ulcerations. The resulting abrasion exposes the dermal fibronectin and collagen which could facilitate the adherence of pathogens such as *V. tapetis* or *A. salmonicida*, aggravate the lesions and subsequently causing ulcerations. This was found in previous studies in other fish species (Wiklund, 1995; Svendsen & Bøgwald, 1997). Skin damage is therefore regarded as a causative agent of skin ulcerations in common dab. Factors that might induce such skin damage (see below) can be regarded as risk-factors for skin ulceration development.

The thickness of the epidermis is correlated with the susceptibility of the skin to damage (Noga, 2000). This fish-related factor can vary with other factors such as sex, age, endocrine status but also with environmental factors such as pH (Iger & Wendelaar Bonga, 1994). The lifestyle of the fish also plays a role. Since common dab are benthic species, commonly making contact with sediment, a thicker epithelium is expected compared to pelagic species. Furthermore, hormones such as prolactin can have an effect in epidermal thickness (Whitear, 1986).

During a small-scale study of common dab epidermal thickness, both on pigmented and non-pigmented side, some seasonal variation was observed with a thicker epidermis in October compared to February (Vroman et al., 2019). This variation was also observed in other species such as brown trout (*Salmo trutta*) (Pickering, 1977) and perch (*Perca fluviatilis*) (Lindesjöö, 1994). More research should be performed to corroborate these results in common dab.

Sex

Contrasting results on the susceptibility of females and males were noted in previous research (Wiklund & Bylund, 1993; Mellergaard & Nielsen, 1997; Lang *et al.*, 1999). No definite proof could be gathered during this research to suggest sex-related difference in susceptibility of skin ulceration development in common dab. Based on the survey, similar susceptibility in males and females was observed (Chapter 7).

Body condition

Although it is not consistently reported, the skin ulcerations in common dab appeared to be correlated with lowered body condition in one study (Mellergaard & Nielsen, 1997). Here, the observational limitations of the surveys are quite clear since we can only observe a correlation and no clear cause-effect relationship. To ascertain the causal effect of a lowered body condition on the susceptibility for ulcer development, an experimental trial should be performed. During our experiments, no decrease of body condition was observed that could be linked to skin ulcer development. However, the experimental situation cannot be compared to a natural situation with major differences in food availability and different potential stressful stimuli.

Length

Based on the data gathered during the four-year survey, larger fish are suggested to be more likely to develop ulcerations, in accordance with previously reported results in common dab (Mellergaard & Nielsen, 1995; Mellergaard & Nielsen 1997; Vethaak *et al*., 2009) and flounder (Wiklund & Bylund 1993). A direct link between the length and susceptibility to develop skin ulcers is difficult to pinpoint as discussed in Chapter 7, however, a more indirect link with fishery-induced lesions can be suggested associated with commercial landing sizes and chances of escaping the net without injury (see below).

9.3.3 Environmental factors

It is remarkable that the two environmental characteristics that are assumed to be impacted the most by climate change (temperature and pH) appear to be related to skin ulceration development in common dab. This might be a warning of the extended impact of climate change on the marine environment. The exact mechanisms behind the correlation with skin ulceration development are not completely resolved.

Temperature

Temperature has been described as an important environmental factor for fish and the entire marine environment. Most reports study the effect of sudden cold or warm shock with extreme temperatures (Donaldson *et al.*, 2008; AnvariFar *et al.*, 2017). This is not believed to be comparable with slow seasonal changes or changes related to climate change, as expected to be relevant in skin ulceration development. Such changes can, nevertheless, cause various changes in all marine organisms and on different levels of biological organization. Changes in temperature can cause physiological changes in the fish skin including apoptosis; furthermore, it can be linked with changes in host susceptibility to develop diseases (Rasmussen-Iyey *et al.*, 2016). Increasing temperature can also affect abundance of bacteria such as *Vibrio* sp. on a global scale (Oberbeckmann *et al.*, 2012; Baker-Austin *et al.*, 2013), production of virulence factors such as cytotoxins and haemolysins (Hamed *et al.*, 2018), increase bacterial survival and transmission rates (Harvell *et al.*, 2002) and it can be important in bacterial adhesion to fish tissue (Hamed *et al.*, 2018).

pH

The alkalinity of the water is not commonly studied but it was stated that even moderate changes in pH can be a substantial stressor for fish (Zahangir *et al.*, 2015). In rainbow trout, exposure to low or high pH caused necrosis and apoptosis in the epidermis which can lead to the development of skin ulcerations (Iger & Wendelaar Bonga, 1994; AnvariFar *et al.*, 2017). *Vibrio tapetis* seems to be able to grow in a wide range of pH's and even has the ability to modify the pH in their environment (Rahmani *et al.*, In prep.). This again emphasizes the complex physiological responses of fish and pathogens to abiotic changes.

Impact of fishing activities

The impact of fishing vessels on the fish has been extensively studied and has been suggested to be related to skin ulceration development in multiple reports (Lüdemann, 1993; Mellergaard & Nielsen 1995; Mellergaard & Nielsen 1997; Mellergaard & Bagge, 1998). All fishing gear types (beam trawl, pulse trawl, otter trawl) involve some degree of mechanical disturbances for fish in different stages of the catch process (Davis, 2002; WG ELECTRA, 2018). All these mechanical impacts ultimately can lead to crushing of the fish, injury, stress, suffocation and ultimately mortality. This again depends on various factors (technical, biological and environmental) of the fishing process, such as mesh size and shape, gear type, water temperature, catch size and composition (Lüdemann, 1993; Suuronen, 2005; Davis & Ottmar, 2006; Veldhuizen *et al.*, 2018). Figure 9.2 provides an overview of all mechanical disturbances and how they can cause skin injury. Importantly, the fishery-induced injuries are most important for non-commercial species such as common dab, species that have a high chance to be discarded (Lüdemann, 1993).

The involvement of fisheries can offer some overarching explanations of various observations of this research. It could explain why skin ulcerations are most frequently observed in common dab, since common dab has higher discard rates compared to sole (*Solea solea*) or plaice (*Pleuronectes platessa*), therefore it can be assumed that relatively more dab with skin injury are re-introduced after catch in the sea. Furthermore, the correlation between length and ulcer development might also be explained by the fishing activities and can be linked to the chances of escaping the net without injury or associated to their size, since dab below 23 cm are commonly being discarded (Seafish, 2010; Miller & Verkempynck, 2016)(Chapter 7).

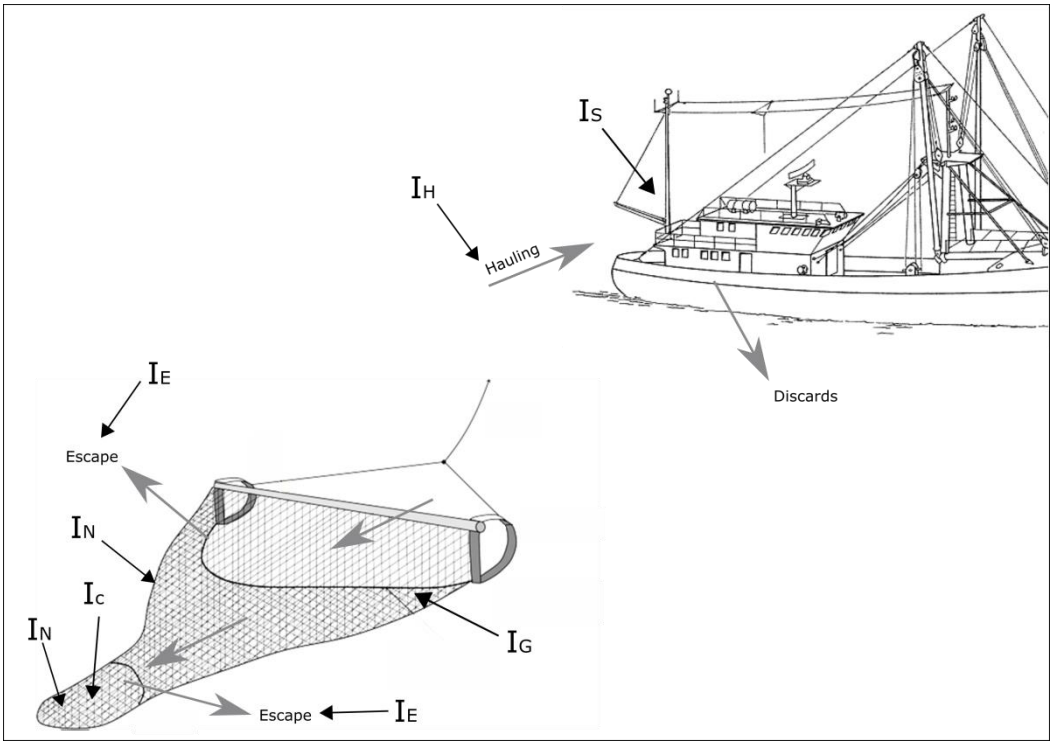


Figure 9.2: Various types of injury due to the mechanical impact in various steps in the fishing process. The light grey arrows indicate the possible routes of the fish (caught in the net, escape, end up in the codend (the outer part of the net where all species are gathered), hauled on board and possibly discarded). The black arrows indicate the types of injuries that can be sustained due to mechanical impact in various steps in the fishing process. I_G: Injury caused by contact with the ground rope, tickler chains, chain matrices, bobbin ropes of conventional trawls or the electrodes of the pulse trawl. I_N: injury caused by the physical contact with the net, this can be both during the passage through the net and in the codend. I_C: injuries caused by other species that are caught. This entails both fish and hard bodied invertebrates such as crabs or stones or suspended sediment particles. I_E: injuries caused by escaping through the meshes of the net, this largely depends on the mesh size and shape. I_H: injuries caused by hauling of the net to the surface resulting in changes in pressure and temperature, but also crushing of fish. I_S: injuries caused by emptying the net on board and sorting through the catch. (Priede, 2017; WG ELECTRA, 2018; FAO, 2019b).

These hypotheses are very difficult to study in the field based on the lack of control, the need of a net to catch the fish and the problem of escaping and therefore undetected injury development (Davis, 2002).

9.3.4 Multifactorial etiology confirmed

One general and clear conclusion from this research is that skin ulcerations indeed have a multifactorial and complex etiology, involving pathogenic agents, fish-related characteristics and environmental factors.

Nevertheless, based on the research performed, we believe that skin damage and subsequent bacterial colonization can be regarded as the ultimate and proximate cause of skin ulcerations, respectively. Evidently, all elements that can cause damage to the skin can be assumed to be risk-factors involved in skin ulceration development. Other factors that were pinpointed to be related to skin ulcer development such as length, fishing intensity, temperature, pH and body condition are

regarded as potential risk-factors that might directly or indirectly change the susceptibility of the fish for development of skin ulcerations in common dab. Figure 9.3 tries to visually represent all factors that were found to be involved in the lesion development in skin ulcerations in the Belgian part of the North Sea. These results are evidently limited to the factors that were included in this research. Other factors that were not recorded, such as the immune status of the fish, might also play a role in the development of skin ulcerations, however, since they were not studied, their role cannot be discussed.

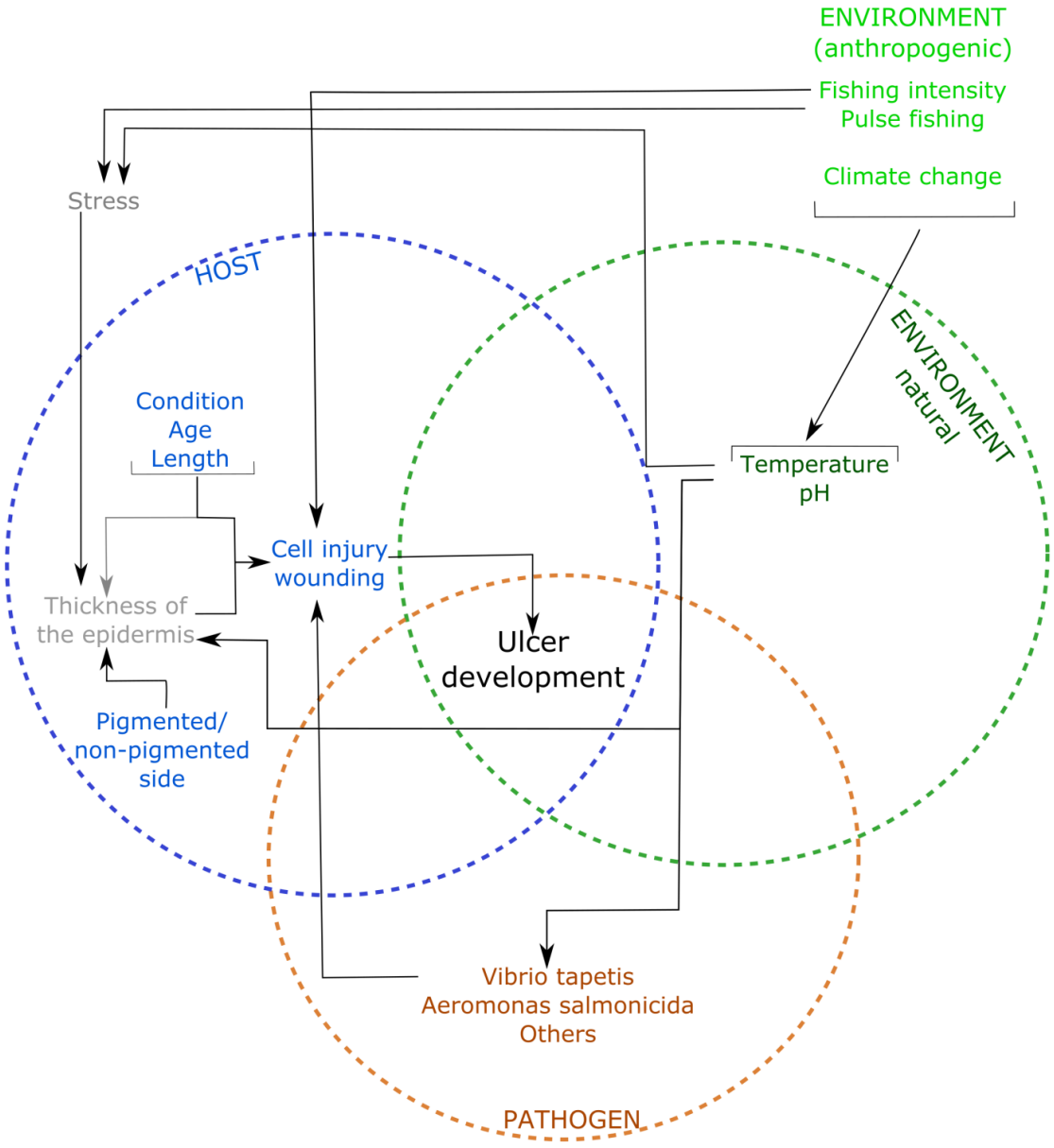


Figure 9.3: Visualization of the pinpointed etiological and risk-factors involved in the development of skin ulcerations in common dab, based on the results of this research.

9.4 Extrapolation of the research results

9.4.1 Extrapolation to other species

The conclusions formulated during this research are based on common dab as a model species. However, at this point, insufficient evidence is present to draw any conclusions to what extent or for which characteristics extrapolation between species is possible. As common dab, sole and plaice are also benthic organisms that are common in the Belgian part of the North Sea. Based on the data gathered during the four-year monitoring survey in the Belgian part of the North Sea on board of the RV Simon Stevin, in total 6067 plaice and 3257 soles were caught of which only 2 plaice were found with an active skin ulceration, resulting in a prevalence of 0.03 % for plaice and 0 % for sole, this in contrast with the 1.3 % prevalence found in common dab (Chapter 7). This might suggest species specific differences in susceptibility for the development of skin ulcerations. An explanation for these observed differences can be, amongst others, the following.

As learned from the research performed in the Norwegian Veterinary Institute, the A-layer of *A. salmonicida* might display a strong predilection towards certain fish hosts (Gulla *et al.*, 2019). The *A. salmonicida* type found in the skin ulcerations of common dab, as described in Chapter 3, possessed a previously undescribed A-layer type, type 15 (Figure 9.1). It is most closely related with an *A. salmonicida* strain isolated from halibut (Singleton; *Hippoglossus hipoglossus*) (microreact.org/project/r1pcOAx9m). Although isolates from other Pleuronectiformes such as turbot (A-layer type 17), plaice (A-layer type 23) and European flounder (A-layer type 18) all seem quite similar (Figure 9.1) (microreact.org/project/r1pcOAx9m). Therefore, to some extent, species-specificity is expected for *A. salmonicida*, although it is unknown if for example it would be impossible for an *A. salmonicida* isolate with A-layer type 18 (isolated from European flounder) to infect common dab. For *V. tapetis*, host specificity could not be pinpointed, although a clear distinction could be made between isolates that were able to induce brown ring disease and isolates that are not (Chapter 5). Nevertheless, *V. tapetis* was hitherto never isolated from other marine flatfish species except from halibut (Chapter 3).

Sole and plaice are economically more interesting for the fishing industry compared to common dab (FAO, 2019a). If we assume that a large part of skin lesions is caused by fishing gear and know that sustained wounds can be active for several hours up to days (Lüdemann, 1993). The only way to develop a skin ulceration is by being released back in the water with an open wound which can get infected and evolve into a skin ulcerations. The main difference between sole, plaice and dab is that the latter has a higher chance to be discarded (WGNSSK, 2019). For example, van Marlen and colleagues (2014) reported that large beam trawlers discard on average 74 kg/h common dab whilst landing only 12 kg/h. In contrast, sole and plaice will be kept on board, killed and sold. Based on this, the chances of encountering a common dab with skin ulcerations are higher compared to encountering a sole or plaice with similar lesions.

9.4.2 Extrapolation to other geographical regions

In some regions, prevalence of skin ulcerations did also increase around 2011, the same period as the observed increase in prevalence in the Belgian part of the North Sea. Baltic cod in the Arkona Sea and Polish sea did increase in prevalence in 2011, the increase was present until 2013 (WGPDMO, 2011; WGPDMO, 2013). A similar increase was observed in common dab in the German Bight (WGPDMO,

2012). Common dab showed an increased skin ulceration prevalence in Morecomb Bay and the Irish Sea in 2011, although the increase in the Irish Sea was already present since 2009 (WGPDMO, 2009). Generally, a decreasing trend in skin ulceration is observed since 2015 in all areas monitored by ICES (WGPDMO, 2018).

Although this study focused only on the Belgian part of the North Sea, the results are believed to be highly relevant for the whole North Sea and worldwide marine habitats with a warning on the effects of climate change and anthropogenic activities. It is difficult to predict the extent of climate change in the ocean and its impact on the prevalence of diseases, but a global impact can be expected.

9.5 Impact of the skin ulcerations

9.5.1 Impact on the fish

Although studying the impact of skin ulcerations on the health of the fish falls outside the scope of this PhD research, it seems reasonable to elaborate on this subject based on the experience gathered in the present research.

As mentioned before, the epidermal integrity is of vital importance for the fish and acts as a physical and immunological barrier between the fish and its aquatic environment (Noga *et al.*, 2000; Rakers *et al.*, 2010). Despite the presumable far-reaching effects of any breach of the skin barrier on the health of the fish, this is only seldom the main topic of a research study. In the Fish Disease Index (FDI), skin ulcerations were evaluated to have the third highest impact on the health of the fish (Lang & Wosniok, 2008). Reported effects of skin ulcerations range from lowered feeding response (Vilar *et al.*, 2012) and changed behavior (Davis & Ottmar, 2006; Vilar *et al.*, 2012) to death (Mellergaard & Nielsen, 1997). Furthermore, Noga (2000) mentioned the increased cost of locomotion due to impairment of mucus production and changed body coloration which can increase fish susceptibility to predation. Most impacts are believed to affect fish energetics and cause a metabolic cost, linked to repair, osmoregulation or increased immune response. This can result in growth impairment, lowered body condition (as observed in chapter 7), reduced fecundity and increased susceptibility to other diseases (Noga, 2000), possibly resulting in mortality.

During the experiments using *V. tapetis*, various fish died following inoculation, this might raise some questions regarding the mortality rates at sea. Although the fish disease monitoring is targeting low-mortality diseases, no information is available on the actual expected mortality rates in common dab (Vethaak & Ap Rheinallt, 1991). If the mortality rates are higher than expected, the observed prevalences of skin ulcerations during field campaigns will be an underestimation of the actual prevalence.

During the experiment, two options were formulated as possible causes of death following experimental infection, being septicemia or osmotic disbalance. Since no evidence could be found suggesting septicemia during the experimental infections (Chapter 4 and 6) we assumed the latter, the most commonly discussed impact of a breach of the skin barrier. Since marine fish reside in a hyperosmotic environment (1000 mOsm/kg) compared to their body fluids (250 - 500 mOsm/kg), they continuously have to deal with passive water loss. To compensate, the fish will drink between 10 % and 20 % of their body weight per day. As a result they take up extra salts and risk hypernatremia and hypochloremia. To prevent this they actively remove Na^+, Cl^+ and K^+ from their

body, mainly by specialized, mitochondria-rich chloride cells in the gills (Greenwell *et al.*, 2003; Ruiz-Jarabo *et al.*, 2015). Due to the scaly epidermis and the mucus coat, not much water will be lost though intact skin. However, an epithelial wound disrupts this barrier and could result in water loss increasing the risks of dehydration (Greenwell *et al.*, 2003; Olsen *et al.*, 2012). In Atlantic herring (*Clupea harengus*), increased plasma osmolality and ion concentrations were observed indicating water loss, however, no solid evidence could be provided that this water was lost through the damaged skin (Olsen *et al.*, 2012). An increased mobilization of blood glucose, a typical direct effect of glucocorticoids, was also observed presumably linked to compensate the energy expenditure from water loss. Furthermore, de-scaling has been reported to induce a stress response in various species (Gadomski *et al.*, 1994; Zydlewski *et al.*, 2010). These indirect effects might initiate a lethal spiral with increased stress response resulting in detrimental effects including glucose exhaustion and increased susceptibility to infections, which ultimately can lead to mortality (Olsen *et al.*, 2012).

It is clear that skin ulcerations can have an effect on the health of the organism. All this knowledge is however based on fish larger than 15 cm. In the *in vivo* experiment, fish with a minimal length of 17 cm were used. In the monitoring surveys, skin ulcerations were absent in any fish smaller than 15 cm. In the international ICES fish disease surveys, only common dab larger than 15 cm are used (Bucke *et al.*, 1996; Lang & Wosniok, 2008). Therefore, the development and impact of skin ulcerations on the smaller fish is somewhat mysterious, with many questions remaining. Two hypotheses can be formulated to explain this lack of skin ulceration observations. First, the chance of obtaining a fishery induced lesion can expected to be lower in smaller fish since we can assume they have more chance to escape the net without injury. Alternatively, but better supported by the literature, it might be that small fish suffer even more from the fishing process resulting in higher mortalities and thus less skin ulcerations observations. The higher mortality might be linked to greater susceptibility to injuries, different barometric pressures, fast fatigue and increased mass-specific metabolic rate resulting in hypoxia (Davis, 2002; Olsen *et al.*, 2012; Benoît *et al.*, 2013). The increased mortality was also found in haddock (*Melanogrammus aeglefinus*) and whiting (*Merlangius merlangus*) smaller than 15 cm and mortality was merely a result of injury and stress not necessarily directly caused by the catch-process (Breen *et al.*, 2007). Furthermore, it is possible that bacterial infection of the injury results in higher mortalities in smaller fish compared to larger fish.

9.5.2 Economic and ecological significance

Besides the impact on the fish's health, some notes can be made on the presumed economic and ecological impact of skin ulcerations. As seen in the surveys, sole and plaice, the most important economic species, have very low to no occurrences of skin ulcerations. Extrapolation of the results of common dab to sole or plaice seemed questionable (see 12.4.1). For now, we can state that common dab is the species that is mainly impacted and therefore the economic impact is estimated to be low.

Common dab is an ecological important species because of its abundance but also because of its important role in the marine food web as both prey and predator (Seafish, 2010). Decreases of the stocks of common dab might have a higher impact on the marine ecosystem due to these food web connections. In the International Bottom Trawl Surveys, providing a robust indicator of changes in stock sizes, common dab stock in the North Sea appeared to be stable or increasing the last years with some recent decreases in 2011 and 2013 but again an increase in 2014 (Hinz *et al.*, 2005;

WGNEW, 2013; WGNSSK, 2015; Miller & Verkempynck, 2016). However, regular stock assessment, as performed for sole and plaice, is not straightforward for bycatch species since it is mainly based on official landings (WGNSSK, 2015).

9.6 Retrospection on the rise in prevalence of skin ulcerations in 2011

A sudden increase of skin ulceration prevalence, as seen in the Belgian part of the North Sea between 2011 and 2014, might indicate a change in the environment. We would like to speculate on a few hypotheses regarding pathogenic agents and environmental risk-factors that might have been involved in this sudden increase in prevalence of skin ulcerations.

9.6.1 Introduction of *V. tapetis* via clams

Oceans are warming and this can cause a geographic re-distribution of waterborne infectious agents such as *Vibrio* sp. on a global scale (Baker-Austin *et al.*, 2012; Oberbeckmann *et al.*, 2012). Furthermore, other marine organisms can also re-distribute due to ocean warming or other stimuli. The Manila clam, the presumed main target of *V. tapetis* causing BRD, has recently been reported for the first time along the Belgian coastline. Two populations of this clam species were described; one population is found near the military harbor of Zeebrugge (Kerckhof, 2014); the second population is found on 'Klein Strand' in Ostend (Kerckhof, 2016). Based on expert judgment, the populations are estimated to be quite recent with predicted arrival in 2011 and 2012 in Zeebrugge and Ostend, respectively (Kerckhof, 2014; Kerckhof, 2016). Northern movements of both pathogenic agents and their host species might have introduced (pathogenic) *V. tapetis* in the Belgian territorial zone. The habitat preference of the clams does not show large overlap with the habitat of common dab. Clams prefer regions in the intertidal zone with lower current velocities, depth and salinity (Bidegain *et al*., 2015). However, *V. tapetis* was also found in other bivalve species such as common cockle and thus might be able to survive in other species which habitats do overlap with common dab. Many questions regarding transmission and host specificity of *V. tapetis* remain.

9.6.2 Changes related to fishing

As mentioned before, the fishing industry was suggested to play a role in the development of skin ulcerations. Their impact can be subdivided in an impact related to the fishing technique and/or related to fishing intensity.

Fishing technique – pulse fishing

The fishing industry is continuously changing to improve the sustainability of their activities. An example is the VALDUVIS project amongst the Belgian fisherman (Valduvis, 2019), searching for alternatives to decrease their impact on the marine ecosystem. One of the promising alternatives that met both the fisherman's aspirations and the need for a more ecofriendly fishing technique is the use of pulse trawl as an alternative to the conventional beam trawl (Soetaert *et al.*, 2016). In 2006, the EU has allowed further employment of this technique by approving an exemption of licenses for 5 % of the fleet of each member state (WGELECTRA, 2018). The exemption of the licenses in 2011 has led to an increase of fishing vessels equipped with pulse trawling gear between 2011 and 2013 (Figure 9.4) (Vansteenbrugge *et al.*, 2020). In 2014, the number of licenses was doubled again resulting in 84 vessels with an exemption. Due to the concurrence of pulse fishing and a sudden

increase in skin ulceration prevalence (Devriese *et al.*, 2015), a cause-effect relation was suggested by some local fishermen. Therefore, this cannot be neglected in this research.

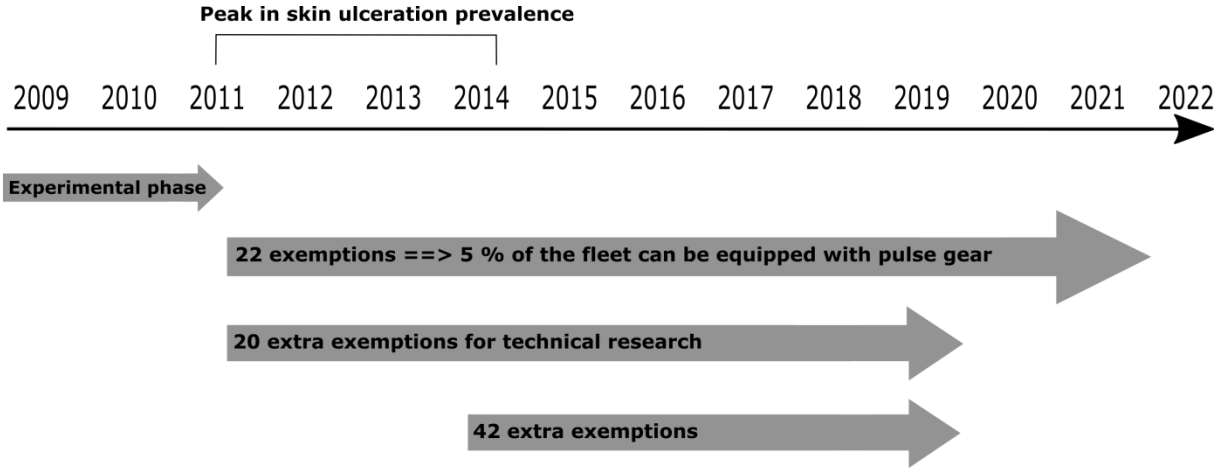


Figure 9.4: Timeline on the exemption licenses to use pulse gear and peak in skin ulcerations.

Nonetheless, the use of this pulse trawl remains controversial due to the unknown impact on the marine organisms that are exposed to this electrical stimulation. Since many questions remained on the impact of this electrical stimulation on the ecosystem, the alternative fishing technique will be banned in the EU in July 2021 (Sandra *et al.*, 2019). Nonetheless, a lot of research has been performed to estimate this influence and explore the safety ranges of this alternative technique (Desender *et al.*, 2016; Soetaert *et al.*, 2016). We will focus on the described direct effect of electrical pulse stimulation on fish and bacteria. A more comprehensive overview of all research results on the impact of electrical pulse stimulation on the marine ecosystem can be found in the ICES report of the working group ELECTRA (WGELECTRA, 2018).

In fish, the external electrical stimulation can affect both the nervous system and the muscles and interferes with normal functioning by inducing action potentials in both (Soetaert *et al.*, 2019). The response of common dab, and other flatfish species, on the electrical stimulation is a contraction of the dorsal musculature leading to a muscular cramp, often described as a U-shape contraction with tail and head pointing upwards (van Stralen, 2005; de Haan *et al.*, 2015). After exposure, common dab exhibits a startle or escape response (Soetaert *et al.*, 2013; de Haan *et al.*, 2015; Soetaert *et al.*, 2016). Experiments using common dab showed that exposure to an electric stimulation did not result in the development of macroscopic skin injuries or increased mortalities that could be directly attributed to the exposure to an electric current (de Haan *et al.*, 2015). During this PhD research some monitoring surveys were conducted in specifically selected locations in the Belgian part of the North Sea where we knew that a commercial pulse trawl has fished the week before. On these surveys, no association could be found between the presence of a pulse trawl and the prevalence of skin ulcerations (Vercauteren *et al.*, in prep). Furthermore, when we would assume a direct cause-effect relationship, we might not expect a decrease in skin ulcerations after 2014, the year where the licenses of Dutch fishermen for pulse trawling have been doubled. No research so far has gathered any proof that the pulse trawling can directly be linked to skin ulceration development.

In contrast to the elaborately studied impact of electrical pulse fishing on the fish, no information whatsoever is available on the possible impact of these electrical pulses on the prokaryotic micro-organisms that are so plentifully present in the marine ecosystem. In other research fields, the use of pulsed electric fields is better developed ranging from degrading of water pollutants (Ghasemi *et al.*, 2019), to biofouling (Schoenbach *et al.*, 2002), human medicine (Dev *et al.*, 2000) and microbial food safety (Dutreux *et al.*, 2000; Barba *et al.*, 2015; Liu *et al.*, 2019). These are all based on the principle of electroporation of cell membranes leading to increased permeability of the cell, which can result in the inactivation of the microorganism (Schoenbach *et al.*, 2002; Barba *et al.*, 2015). In contrast, Manabe and coworkers (2016) found increased expression levels of virulence factors of *V. parahaemolyticus* using pulsed electric fields. Although it might be tempting to extrapolate these results to the electricity-microbe interaction in pulse fishing, the characteristics (voltage, exposure time) of the pulse fields used are too different to allow any extrapolation (Barba *et al.*, 2015; Soetaert *et al.*, 2019). Research is necessary to elucidate the effect of pulse fishing on the microbial communities in the marine ecosystem.

Mainly all studies on the impact of pulse fishing, focus on the effect of the electrical stimulation as a tool to force the flatfish to leave the sediment and guide them into the nets. However, as described before, injuries caused by mechanical or electrical stimulation are only one potential source of injuries (Figure 9.2).

Nonetheless, the total physical impact on the fish is estimated to be lower compared to beam trawl fishery (Davis, 2002; Uhlmann *et al.*, 2016; van der Reijden *et al.*, 2017). This is mainly linked to lower bycatch (less hard-bodied invertebrates), quicker sorting on board, lower fishing speed and reduced damage due to the stimulation (Davis, 2002; Uhlmann *et al.*, 2016; van der Reijden *et al.*, 2017). Moreover, the discarded fish have a higher chance to survive when caught using a pulse trawl in comparison with a beam trawl (Uhlmann *et al.*, 2016). Thus if we take the total fishing process into account, one might hypothesize that fish with injuries are still introduced in the sea after catch (although in lower numbers compared with conventional beam trawling), however due to the increased survival, fish that were caught using the pulse trawl have more chance of survival and thus more chance to develop skin ulcerations.

Fishing intensity

Since injuries can be sustained during the fishing process, higher fishing intensity (i.e. the number of vessels per unit area per unit of time (Sanders & Morgan, 1976)) might introduce more fish with injuries in the marine environment, increasing the prevalence of skin ulcerations.

Between 2006 and 2018, the Belgian fishing intensity (hours at sea; beam trawling) has decreased drastically, both in the large fleet (> 221 kW) and in the small fleet (≤ 221 kW). Part of the decrease (2009 - 2011) has been linked to the fuel crisis in 2008. The Dutch fleet showed the same decrease but their fleet increased again in 2011 with the implementation of pulse fishing. The small segment of the Dutch fleet (both pulse and beam trawl) has moved southwards around 2010 hereby increasing the fishing intensity in Belgian territorial waters (12 nm) (Figure 9.5) (Turenhout *et al.*, 2016; Vansteenbrugge *et al.*, 2020). The Dutch large fleet segment moved to the North and West, with reduced fishing intensity in the Belgian territorial waters (Vansteenbrugge *et al.*, 2020).

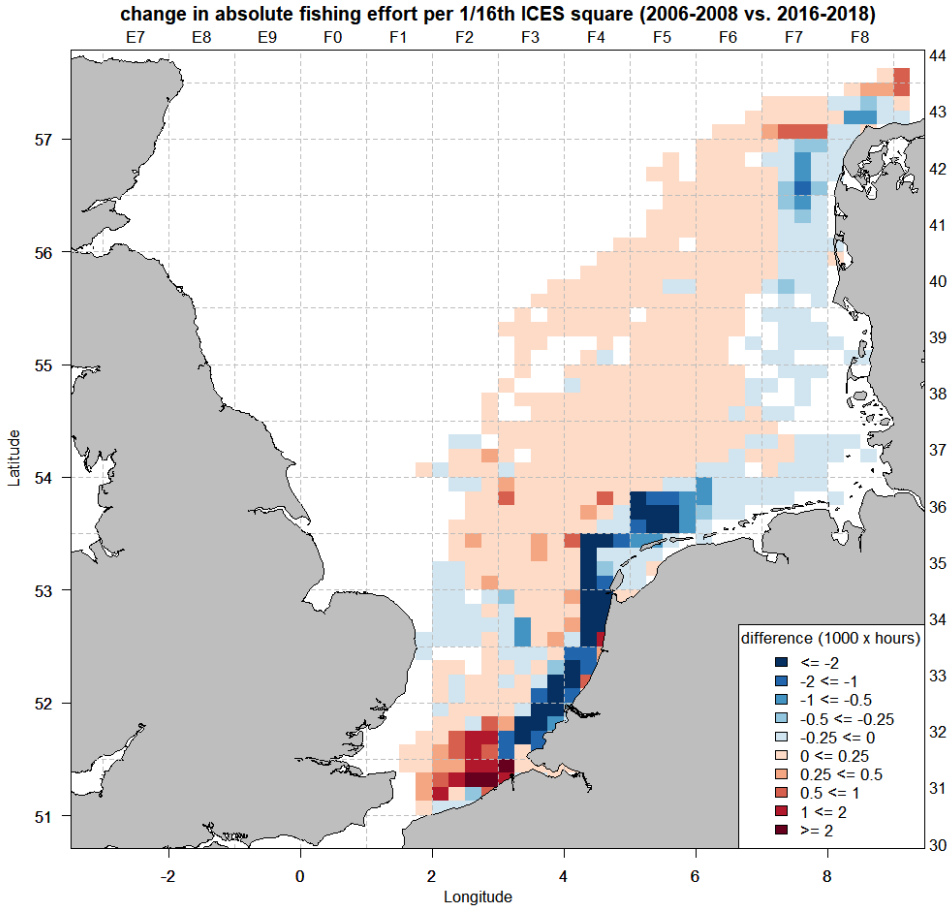


Figure 9.5: The absolute difference in fishing intensity in the North Sea between 2016-2018 and 2006-2008 for the Dutch small fleet. Red and blue indicate an increase or decrease, respectively.Image retrieved from Vansteenbrugge et al. (2020).

The geographic shift between 2006 and 2018 in fishing effort of the Dutch small fleet fishing vessels, resulting in higher fishing pressure in Belgian territorial waters might (partially) explain the changes observed in skin ulceration prevalence. Nevertheless, the general fishing intensity in the Belgian part of the North Sea showed a decrease between 2009 and 2010 with a sudden increase in 2011 which also can partly offer an explanation (Vansteenbrugge *et al.*, 2020). This suggests the combined effect of fishing intensity and technique (the rise in pulse fishing in the Dutch fleet), amongst others, in the increase in skin ulceration prevalence in common dab in the Belgian part of the North Sea between 2011 and 2014.

9.7 Implications on policies

The overall aim of this research was to increase the scientific understanding of skin ulcerations and possible factors that are linked to their development. Although it was not an objective of this study, the results might guide some of the current national and international policies. In the European Union, the Marine Strategy Framework Directive (MSFD) was put in place to protect the marine ecosystem (2008/56/EC) and aims to achieve a good environmental status by 2020, guided by 11 quality descriptors, focusing on biological biodiversity, fisheries, food webs, marine litter and environmental contamination, amongst others (Giltrap *et al.*, 2017). In this framework, fish diseases are regarded as a biological effect associated with contaminants (Descriptor 8: "contaminants must not be detected at concentrations that give rise to pollution effects"). However, based on the results gathered in the present study, mainly based on the four-year survey, no link could be established between the traditionally monitored sources of pollution (polychloorbifenyl (PCB), heavy metals) and the presence of skin ulcerations in the Belgian part of the North Sea (Chapter 7). More research would be necessary; however, an indirect effect or effects of non-monitored contaminants are still possible.

Nevertheless, the results of this research clearly indicate an impact of fishery on the development of skin ulcerations, emphasizing on the need to work on the sustainability of the fishing industry. When aiming for an ecosystem-based approach, development of technical alteration should not only be focused on length and species selectivity to increase economic value of the catch but also to reduce the effects on the ecological important bycatch species. This will require more research, although some progress has been made regarding improvements of the fishing gear (such as maze size, type, escape panels) (Fonteyne & Polet, 2002; Van Craeynest *et al.*, 2013; Vanderperren *et al.*, 2014; Valduvis, 2019). All measures that can reduce bycatch, fishing-gear related injuries or improve the sorting process on board and thus all measures that can reduce the chances on fishery-related injuries, can offer opportunities to reduce the ecological impact of this sector. The fishing industry already shows willingness to increase the sustainability of their profession, in collaboration with scientists, as proven in the VALDUVIS project (Valduvis, 2019). Evidently, the guidelines to improve sustainability should be based on scientific knowledge on the causes and risk-factors involved in disease development (not only skin ulcers but also other diseases), urging for more scientific research to substantiate this sustainable development and ensure a good environmental status of the marine environment.

9.8 Recommendations for further research

Despite all studies and international surveys in the past, the etiology of skin ulcerations in common dab remained unknown. Moreover, the sudden and inexplicable increase in prevalence of skin ulcerations between 2011 and 2014 in the Belgian part of the North Sea accentuated the need in understanding its cause (s). During this PhD study, new insights were gained in the multifactorial etiology of skin ulcerations. However, more information is warranted on its contributing pathogens, fish-related factors and environmental factors.

The quest for **pathogens** involved in the development of skin ulcerations needs to be continued. The use of culture-independent bacteriological methods such as DNA metabarcoding using Next Generation Sequencing can aid in this search by gaining a more complete overview of pathogens that are present in the skin ulcerations and/or in various stages of skin ulceration development. These techniques should be employed during field campaigns and the role of identified bacteria can be further confirmed in experimental trials as proposed in Chapter 4 and 6. Such high throughput sequencing methods might also be used in an analysis of the microbiome of the fish, whereby a comparison between the microbiome of healthy fish and fish with skin ulcerations might gain some insights in the susceptibility of fish for skin ulceration development and the involvement of the microbiome in this process.

The unique characteristics of the two isolated bacterial species, *V. tapetis* and *A. salmonicida*, in skin ulcerations in common dab, raised questions on the specificity of these strains and the possible transmission of bacteria between hosts and species. First steps were taken to evaluate the specificity of *V. tapetis* strains by studying differences in clam pathogenic and non-pathogenic isolates. More in-depth analysis of the protein profile of the bacteria and especially further characterization of the 6294.76 kDa protein could be performed to elucidate the host-specificity. This can be done using mass spectrometry (McHugh *et al.*, 2008). *In vivo* infection studies in both fish and clam using different *V. tapetis* strains might also provide information regarding host-specificity.

After experimental infection (Chapter 4 and 6), a different course of the disease was observed, possible indicating a distinct pathogenesis. To study this, as well as possible interactions between both bacteria, the two-chamber skin explant model might offer a valuable tool. It allows examination of different infection protocols in a standardized and repeatable manner. Furthermore, since muscle is present in the model, deeper invasion of bacteria as observed in *in vivo* studies and the reported muscle preference of A*. salmonicida* can be studied.

An intriguing fish-related risk factor associated with the development of skin ulcerations is the size of fish, whereby none of the fish smaller than 15 cm was observed with a skin ulceration. Not only field studies (eg sea surveys) but also *in vivo* experiments are needed to investigate skin ulceration development in smaller fish. Similar infection studies as described in Chapter 4 and 6, using smaller fish, would be useful to t obtain more information on the development of skin ulcerations and their impact. Furthermore, the effect of stress as an immunosuppressing factor could result in an increased susceptibility for skin lesion development. The lack of a reliable method to measure chronic stress in fish has hampered research in this field of interest. However, determination of scale cortisol is considered as a promising tool (Aerts et al., 2015). During this PhD research, we have demonstrated that common dab is able to accumulate cortisol in its scales. This seems to occur proportionally to increased circulating concentrations of plasma cortisol after 30 days of feeding on cortisol-spiked feed. In this study, no clinical health effects were associated with increased cortisol concentrations (data not shown). The association between increased cortisol and ulceration development needs to be studied in detail together with seasonal variations in scale cortisol concentrations, the effect of pigmentation on accumulation of cortisol and the effect of stressors for the fish. If the accumulation of cortisol in the scales appears to be ecologically relevant, it might be useful to include this in the monitoring for health of the entire marine ecosystem.

Further research regarding the role of **environmental and anthropogenic risk factors** in skin ulceration development can be conducted in two phases. First, the correlations that were found during the four-year survey can be experimentally verified to establish causality. The developed skin explant model, can aid in the study of the effect of sea water temperature and pH. Furthermore, other environmental factors can be included by keeping various skin explants in different conditions. The subsequent inoculation of the pathogens described above, could contribute in getting new insights in the pathogenesis (integrated approach).

Secondly, since we have demonstrated that pre-existing lesions predispose fish to skin ulceration development, a precise estimation of the factors causing abrasion in flatfish is warranted. One factor that can be examined is the effect of the fishing gear in the development of abrasions. Not only grossly visible skin damage, also subtle lesions should be recorded. For this, we have demonstrated that fluorescein can be useful (data not shown). Estimation of a correlation between size, number or type of lesions with various characteristics of fishing gear (mesh size, mesh shape, net material,...) could aid in designing fishing gear eventually resulting in less skin lesiosn in discarded fish. This could contribute to a more sustainable fishery; however a large scale field evaluation will be mandatory.

Thirdly, concerns were raised on the role of electrofishing in the development of skin lesions. During our four-year survey, no data were obtained that demonstrated a possible impact of pulse fishing on skin ulceration development. Therefore, *in vivo* experiments using animals exposed to electric pulses in similar infection models as described in chapter 4 and 6 can be performed. The direct effect of the electrical stimulus on the bacteria and/or skin can also be studied using *in vitro* methods such as the explant model. The results of such experiments can be extremely valuable in the ongoing political and scientific discussion regarding the impact of pulse fishing on the ecosystem.

9.9 General conclusion

Concerns about the sudden change in disease prevalence, expressed by both the scientific and sea-guarding communities, were the direct trigger for this research. By combining different experimental approaches and various research results, we can conclude that skin ulcerations have indeed a multifactorial etiology with involvement of pathogenic agents, fish-related factors, environmental and anthropogenic parameters. Skin abrasions are a major contributing factor in the disease development and lesions can subsequently be invaded by pathogens such as *V. tapetis* or *A. salmonicida* resulting in a skin ulceration. Various fish-related (length condition), environmental (temperature, pH) and anthropogenic (fishery) risk-factors can affect the susceptibility of the fish to develop skin ulcerations. With the innovative two-chamber skin explant model developed during the present research, future integrative studies are made possible studying the combined effect of various factors on skin ulceration development.

Supplementary files

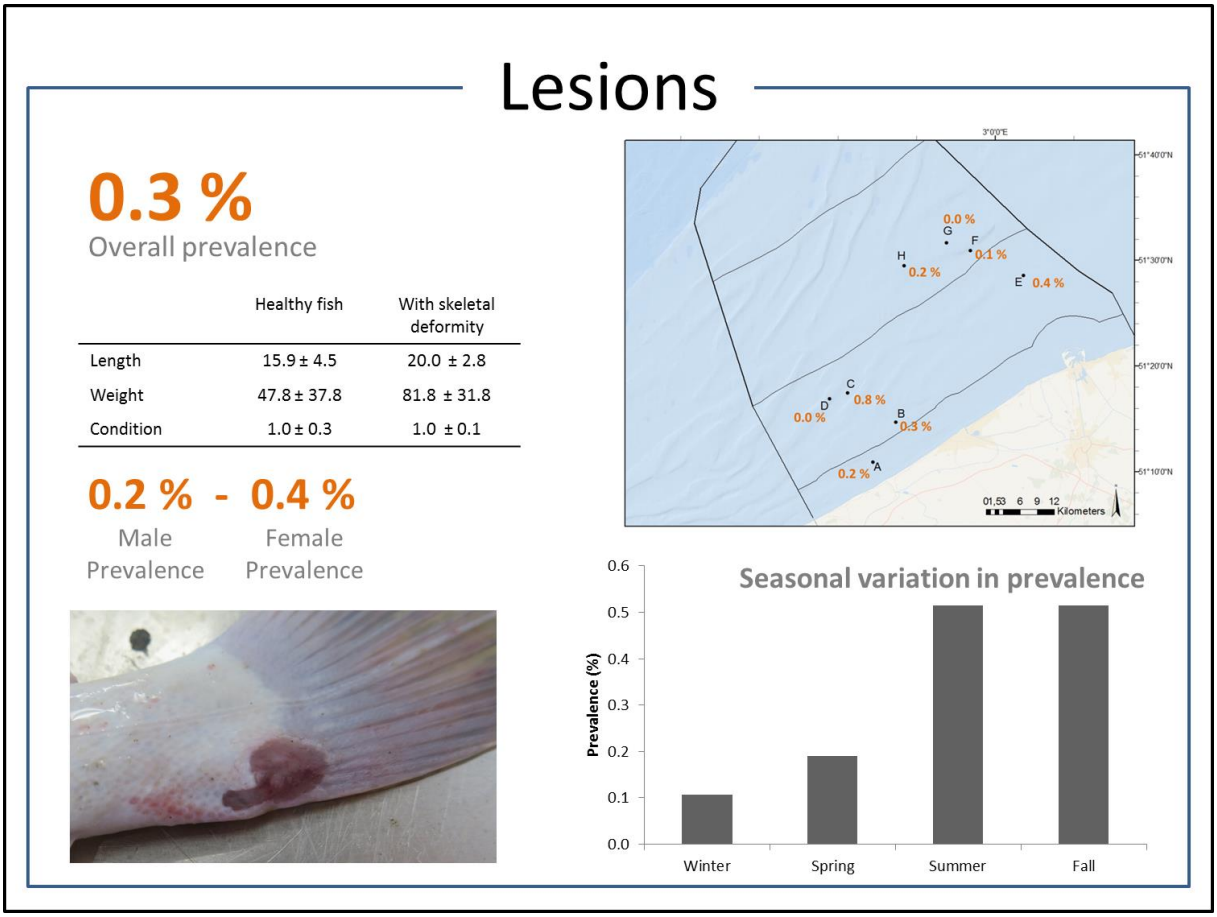
Supplementary file 1

Scoring of the different parameters that are part of the "Gross Ulceration Score" (GUS). The GUS is the sum of the score of all the parameters indicated below and represents the severity of an ulceration.

Parameter	Score	Description
Pigmentation inside or around the ulceration	0	No dark pigmentation visible in/around the ulceration.
	1	Dark pigmentation visible in half of the ulceration.
	2	Dark pigmentation visible in the complete ulceration.
	3	Dark pigmentation visible in the total ulceration with high intensity of the pigment.
Elevation of the edge of the ulceration	0	No elevation of the edge of the ulceration.
	1	Elevation of part of the edge of the ulceration. Only certain parts of the edge are elevated.
	2	The total edge of the ulcerations is elevated.
Healing around the lesion	0	White edge present around the ulceration.
	1	White edge present around part of the ulceration.
	2	No white edge present around the ulceration.
Shape of the ulceration	0	No clear edge of the lesion visible. The ulceration merges seamlessly with the surrounding healthy tissue.
	1	The ulceration has an irregular shape.
	2	The ulceration has a rounded shape with a clear edge.
Hemorrhages around the ulceration	0	No hemorrhages or discoloration visible around the lesion.
	1	A small number of petechiae visible around the ulceration.
	2	The whole ulceration is surrounded by mild hemorrhages.
	3	Around the lesion a dark red or purple discoloration is visible, resembling a heavy subepidermal hemorrhage.
Color of the ulceration itself	0	The ulceration has a rather normal color, no pink/red discoloration visible. Be careful, a lack of pigmentation is possible.
	1	The ulceration has a lightly pinkish color.
	2	The ulceration is red.
	3	The ulceration is deep red, resembling active ulceration and intense bleeding.
Depth of the ulceration	0	There is no ulceration visible.
	1	The upper epidermal tissue is missing, recognized by the absence of scales and pigmentation.
	2	Epidermal and dermal tissue are missing, the muscle is exposed, however the ulceration is quite superficial.
	3	Clear deep ulceration into the muscular tissue.

Supplementary file 2

Overview of the externally visible diseases encountered during the four-year survey, with their prevalence, spatial and seasonal variations.

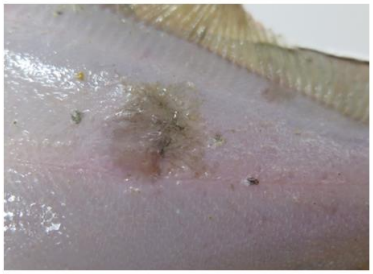


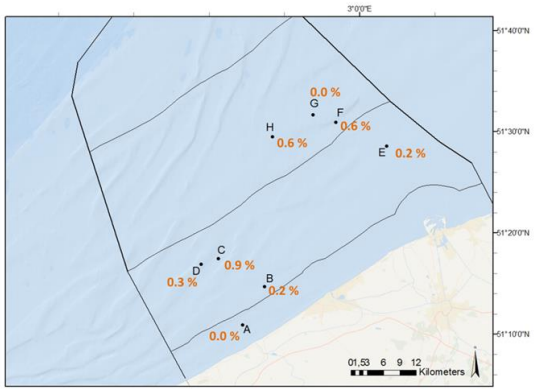
Healed lesions

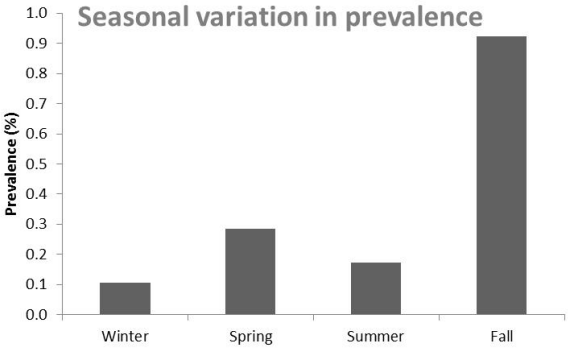
0.4 %
Overall prevalence

	Healthy fish	With healed lesions
Length	15.9 ± 4.5	20.5 ± 2.7
Weight	47.8 ± 37.8	81.7 ± 39.4
Condition	1.0 ± 0.3	1.0 ± 0.3

0.3 % Male Prevalence - **0.4 %** Female Prevalence





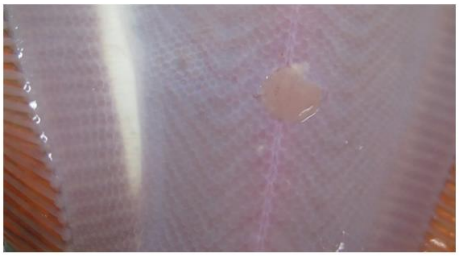


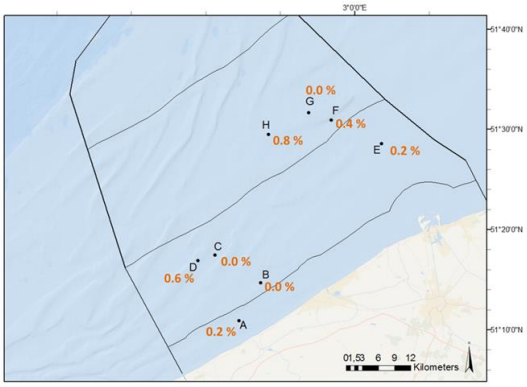
Papilloma-like lesions

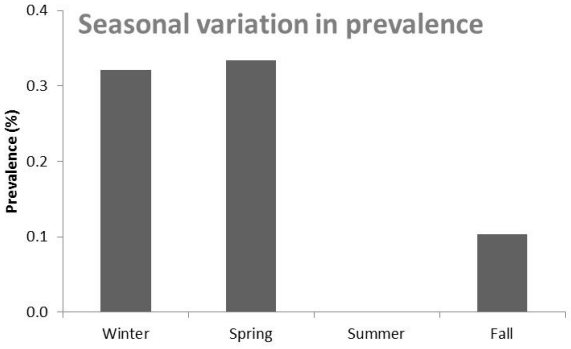
0.2 %
Overall prevalence

	Healthy fish	With papilloma-like lesions
Length	15.9 ± 4.5	19.3 ± 2.6
Weight	47.8 ± 37.8	72.2 ± 39.0
Condition	1.0 ± 0.3	0.9 ± 0.2

0.2 % Male Prevalence - **0.3 %** Female Prevalence





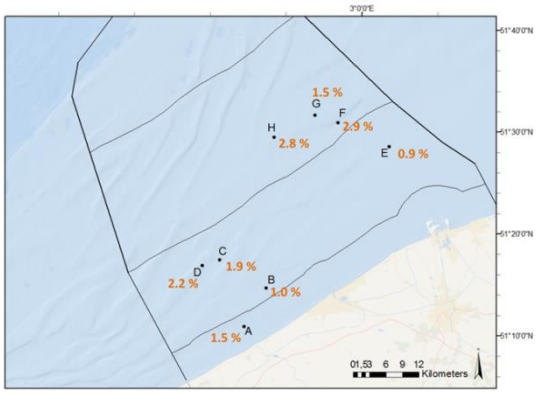


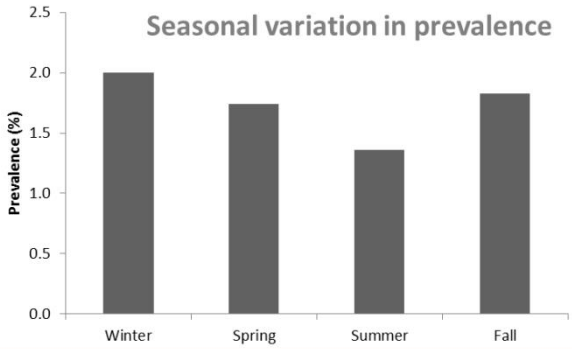
Hyper- or hypopigmentation

1.8 %
Overall prevalence

	Healthy fish	With malpigmentation
Length	15.9 ± 4.5	18.2 ± 3.9
Weight	47.8 ± 37.8	62.0 ± 38.0
Condition	1.0 ± 0.3	1.0 ± 0.3

2.2 % Male Prevalence - **1.6 %** Female Prevalence



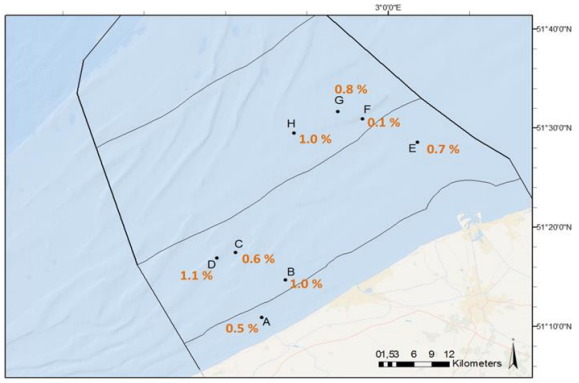


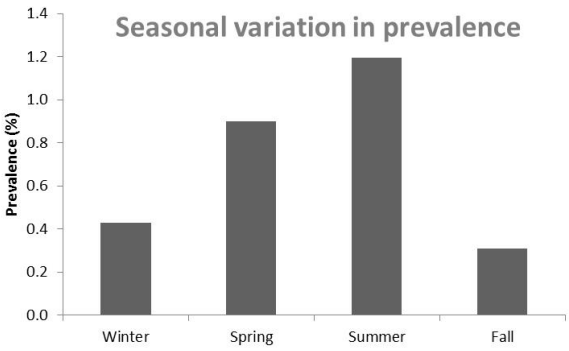
Skeletal deformities

0.7 %
Overall prevalence

	Healthy fish	With skeletal deformity
Length	15.9 ± 4.5	16.2 ± 3.4
Weight	47.8 ± 37.8	53.0 ± 37.6
Condition	1.0 ± 0.3	1.1 ± 0.2

0.7 % Male Prevalence - **0.8 %** Female Prevalence





Supplementary file 3 - Results microplastic analysis

Methods

Microplastics were determined in the stomach of 48 fish in total whereby 65 % (n = 31) had a skin ulceration. Common dab (*Limanda limanda)* of all sampling locations were sampled.

Stomachs of the fish were dissolved in 200 ml 10 % KOH at 60 °C during 24 hours. The dissolved tissue was filtered (12 – 15 µm) and transferred to a Petri dish. Microplastics were visualized using a stereomicroscope. Based on type and color, microplastics were categorized. Using the hot needle test the identification was verified. To minimalize contamination, standard protocols were followed develop during Cleansea (FP7), EcSafeseafood (FP7) and Micro (Interreg-2-Zeeën) projects. All glass equipment was rinsed thoroughly using Type II filtered water.

The results were corrected for the average blanco. A quantification limit (LOQ) was calculated as three times the standard deviation of the procedure-blanco's. When the sample did not contain any microplastic, the reporting limit of 0.1 microplastic per sample was used.

Results

In total, four fish out of 48 fish (8.3 %) contained microplastics in the stomach. Two fish with a skin ulceration contained 6.4 and 9.4 microplastics fibers in their stomach. Two other fish contained 1 granule. All other fish had amounts below the LOQ.

Conclusion

Common dab contained very low amounts of microplastics in their stomach. Due to the low amount of microplastic, drawing conclusions regarding correlations with locations or presence of ulcerations is not possible.

Number of fish	Fiber	Granule	Films	Total	Ulceration	Location
20	<LOQ	<LOQ	<LOQ	<LOQ	Yes	7802
21	<LOQ	<LOQ	<LOQ	<LOQ	Yes	7802
22	<LOQ	<LOQ	<LOQ	<LOQ	Yes	7802
23	<LOQ	<LOQ	<LOQ	<LOQ	Yes	7802
24	<LOQ	<LOQ	<LOQ	<LOQ	Yes	7802
28	<LOQ	<LOQ	<LOQ	<LOQ	Yes	215
29	<LOQ	<LOQ	<LOQ	<LOQ	Yes	215
30	<LOQ	<LOQ	<LOQ	<LOQ	No	215
32	6.4	<LOQ	<LOQ	6.4	Yes	215
33	<LOQ	<LOQ	<LOQ	<LOQ	No	215

35	<LOQ	<LOQ	<LOQ	<LOQ	Yes	7802
36	<LOQ	<LOQ	<LOQ	<LOQ	No	7802
37	<LOQ	<LOQ	<LOQ	<LOQ	Yes	215
38	<LOQ	<LOQ	<LOQ	<LOQ	Yes	215
40	<LOQ	<LOQ	<LOQ	<LOQ	No	215
41	<LOQ	<LOQ	<LOQ	<LOQ	Yes	215
42	<LOQ	<LOQ	<LOQ	<LOQ	No	215
43	9.4	<LOQ	<LOQ	9.4	Yes	215
44	<LOQ	<LOQ	<LOQ	<LOQ	Yes	2252
45	<LOQ	<LOQ	<LOQ	<LOQ	Yes	2252
46	<LOQ	<LOQ	<LOQ	<LOQ	Yes	2252
47	<LOQ	<LOQ	<LOQ	<LOQ	Yes	2252
48	<LOQ	<LOQ	<LOQ	<LOQ	No	2252
53	<LOQ	1.0	<LOQ	<LOQ	No	ftWT1biss
54	<LOQ	<LOQ	<LOQ	<LOQ	Yes	ftWT1biss
55	<LOQ	<LOQ	<LOQ	<LOQ	Yes	ftWT3biss
56	<LOQ	<LOQ	<LOQ	<LOQ	No	ftWT3biss
57	<LOQ	<LOQ	<LOQ	<LOQ	Yes	TB01
58	<LOQ	<LOQ	<LOQ	<LOQ	Yes	TB01
59	<LOQ	<LOQ	<LOQ	<LOQ	No	TB01
60	<LOQ	<LOQ	<LOQ	<LOQ	Yes	215
61	<LOQ	<LOQ	<LOQ	<LOQ	Yes	215
62	<LOQ	<LOQ	<LOQ	<LOQ	Yes	215
63	<LOQ	<LOQ	<LOQ	<LOQ	No	215
64[1]	<LOQ	<LOQ	<LOQ	<LOQ	Yes	215
65[1]	<LOQ	<LOQ	<LOQ	<LOQ	No	215
66[1]	<LOQ	<LOQ	<LOQ	<LOQ	Yes	2252
68[1]	<LOQ	<LOQ	<LOQ	<LOQ	No	2252
70[1]	<LOQ	<LOQ	<LOQ	<LOQ	No	2252
73	<LOQ	<LOQ	<LOQ	<LOQ	Yes	TB01
74	<LOQ	<LOQ	<LOQ	<LOQ	Yes	BRN01

75	<LOQ	<LOQ	<LOQ	<LOQ	No	BRN01
76[1]	<LOQ	<LOQ	<LOQ	<LOQ	Yes	2252
77[1]	<LOQ	<LOQ	<LOQ	<LOQ	No	2252
82[1]	<LOQ	<LOQ	<LOQ	<LOQ	Yes	2252
83[1]	<LOQ	1.0	<LOQ	<LOQ	No	2252
84	<LOQ	<LOQ	<LOQ	<LOQ	Yes	120
85	<LOQ	<LOQ	<LOQ	<LOQ	No	120

Summary

Flatfish (Order Pleuronectiformes) are fascinating species inhabiting marine, estuarine and, to a lesser extent, freshwater habitats. They can be recognized by their flattened body with both eyes located on the same side of the head and associated lateralized swimming posture. Flatfish are gastronomically appreciated and therefore commercially valuable for the fishing industry worldwide. But in the marine ecosystem they also play an important ecological role as both predator and prey species in the marine food web.

The marine ecosystem where flatfish are living is continuously influenced by various human activities such as shipping, fishing, dumping of dredged material and emission of greenhouse gasses. One of the possible biological effects of these activities is the development of diseases such as skin ulcerations, defined as lesions where the epidermis and basement membrane are affected (**Chapter 1**). For an unknown reason, flatfish seem more susceptible for the development of several diseases including skin ulcerations. International standardized surveys have been performed to monitor the prevalence of these diseases in various regions in the North Sea and adjacent areas, and use them as a proxy for the health of the marine ecosystem as a whole. Although the prevalence of skin ulcerations is monitored, the exact causality and risk factors involved in their development have not yet been pinpointed. It might seem straightforward to find a cause for these lesions; however, the opposite is found to be true. Fish and the marine environment are both complex and intimately connected. Furthermore, the sea is highly variable in time and space and it is nontrivial to estimate what is happening beneath the sea surface (**Chapter 1).**

On these regular surveys in the Belgian part of the North Sea, a sudden and inexplicable increase of skin ulcerations prevalence was observed in common dab (*Limanda limanda*) in the period between 2011 and 2014. This triggered us to perform an in-depth research on the etiology (i.e. cause) of this disease.

A general framework states that a disease is mainly linked to an imbalance between pathogens, fish-related characteristics and environmental factors, a detailed description can be found in **Chapter 1**. This framework guided us through the research aiming to increase the scientific understanding of the etiology of skin ulcerations in common dab (*Limanda limanda*) from the Belgian part of the North Sea (**Chapter 2**). An integrated, comprehensive and multidisciplinary research approach was chosen that combines surveys at sea and laboratory experimental studies to disentangle the involvement of pathogenic agents (**Chapter 4, 5, 6 and 7**), fish-related aspects (**Chapter 7**) and environmental factors (**Chapter 7**).

PART I: Explorative study

The research started at sea during a first explorative survey in the Belgian part of the North Sea, organized to examine these skin ulcerations and gather some first indications on the factors that can

be involved in their development (**Chapter 3**). Various fish with ulcerations were encountered and thoroughly sampled. Upon further examination of the ulcers, pure cultures of the bacteria *Vibrio tapetis* and *Aeromonas salmonicida* were isolated. *Vibrio tapetis* is a pathogen causing Brown Ring Disease in clams and was only more recently reported as the cause of infections in fish. However, it was the first time that this bacterial specis was demonstrated in skin ulcerations in wild-caught marine fish. *Aeromonas salmonicida* is a common fish pathogen; however the isolate from dab have an unique, yet previously undescribed, A-layer type. The latter consists of a protein, forming an additional external layer on the cell wall and is believed to protect the bacteria during infection.
This explorative study has proven to be a perfect starting point for further research regarding the etiological agents involved in skin ulceration development.

Part II: Fish-pathogen interactions

To prove the involvement of the above mentioned bacteria in the development of skin ulcerations in common dab, an *in vivo* infection experiment was conducted. Besides investigating the role of the bacteria, the role of the intact skin barrier and the difference between the pigmented and non-pigmented side were investigated. After a bath immersion challenge, the fish were monitored for 21 days in which mortality, clinical signs and ulceration development were recorded. After 21 days, the animals were sacrificed and sampled.

Following infection with *V. tapetis* (**Chapter 4**), high mortality was observed four days after inoculation. Fish that were infected did develop more severe skin ulcerations compared to fish that were sham treated, thus suggesting a role of *V. tapetis* as a causative agent in the development of these lesions. Furthermore, since skin ulcerations mainly developed in the zones were scales and epidermal tissue were removed, in contrast to control or mucus-free zones, prior damage to the skin seems to be a major contributing factor for skin ulceration development.

Further research on the specificity and similarity of *V. tapetis* isolates derived from common dab, other fish species and bivalves was performed (**Chapter 5**). The variability was studied using an *in vitro* virulence test as well as genetic (sequencing of virB4 gene) and protein-based (MALDI-TOF MS) approaches. We found that none of the methods were able to unambiguously discriminate between isolates derived from fish and clam species. Furthermore, all three methods were compared and evaluated in order to find a discriminative tool for pathogenicity against clams or fish, independent of the species they were derived from. The protein-based approach did appear to be able to discriminate between isolates that are pathogenic for clams and those that are not.

After infection with *A. salmonicida* (**Chapter 6**), mortality occurred gradually after 12 days post-inoculation. Most ulcerations developed in the zone where the scales and epidermal tissue was removed, again emphasizing on the important role of an intact skin barrier to protect against bacterial invasion and skin ulceration development. *A. salmonicida* seems to be involved in the development of skin ulcerations in common dab although its relative importance and the underlying pathogenesis still needs to be elucidated.

Part III: Integrated approach

Bimonthly surveys were performed on eight fixed sampling locations in the Belgian part of the North Sea during this PhD study (2016 – 2019). To study the etiology of skin ulceration in an integrative manner, data regarding pathogens, fish-related and environmental risk-factors was collected (**Chapter 7**).

Pathogens: The involvement of *V. tapetis* in skin ulceration development was confirmed since this bacterial species was isolated again from several skin ulcerations. Furthermore, *V. alginolyticus* was also commonly isolated. Other identified genera were, amongst others, *Bacillus* sp., *Psychrobacter* sp., and *Actinobacter* sp., although they were present in only a minority of the ulcerations. *Aeromonas* sp. was also identified in one lesion however no species identification was possible.

Fish-related factors: Most fish had only one ulcer, although up to five ulcerations per fish were observed. Ulcerations were mainly present on the non-pigmented side and were randomly distributed. Larger fish appeared to have a higher chance of ulceration development. Poor condition was associated with the presence of an ulceration. The sex of fish did not influence the susceptibility to develop skin ulcers.

Environmental factors: Our study revealed that temperature and pH were positively correlated with the presence of skin ulcerations, indicating that with higher temperature and pH, more fish will develop skin ulcerations. Furthermore, the fishing pressure was also correlated with skin ulcerations.

Importantly, since this is only an observational study, it does not permit inferring causality of any of the involved factors. In studies described above, the role of *Vibrio tapetis* and *Aeromonas salmonicida* in skin ulceration development has been demonstrated. Further experimental research will be necessary to pinpoint exact cause-effect relationships of the other correlations.

As we have demonstrated in the studies mentioned above, multiple factors can interact and have an effect on the development of skin ulcerations. Therefore, we aimed to develop an innovative *in vitro* model which allows us to study pathogenic agents, fish-related characteristics and environmental factors in an integrated approach. For this purpose, a two-chamber skin explant model was designed envisaging the difference in salinity between the inner and outer environment of marine fish (**Chapter 8**). After 24 h in the model, the explant skin did not show apparent differences with the control skin based on minimal histological changes in structure, number of cell layers, goblet cells and eosinophilic granulocytes. Proliferative and apoptotic rates did also appear to be similar. Only a small increase of epidermal thickness was observed, unrelated to the number of cell layers. This demonstrates that our innovative model is a comprehensive and valuable *in vitro* alternative enabling further research on the causative agents involved in skin ulceration.

This research has focused on various individual aspects of skin ulceration in common dab in the Belgian Part of the North Sea regarding pathogenic agents, fish-related factors and environmental factors. Nonetheless, there are some general points that have to be discussed in light of their combined findings. Therefore, the general discussion (**Chapter 9**) tackles some general points and comprises an overarching discussion on the scientific research methodology and the results. Furthermore it suggests what possibilities the future holds for the research on fish diseases. The

results gathered in this study are used to support retrospection on the sudden increase in skin ulceration prevalence observed between 2011 and 2014 whereby the geographic redistribution of the pathogen *V. tapetis* and the change in fishing technique and/or intensity in the Belgian part of the North Sea are formulated as hypotheses for this increase.

In conclusion (**Chapter 9**), by combining different experimental approaches and various research results, we can conclude that skin ulcerations have indeed a multifactorial etiology. Skin abrasions are a major contributing factor in the disease development and lesions most probably are subsequently invaded by pathogens such as *V. tapetis* or *A. salmonicida* resulting in a skin ulceration. Various fish-related (length, condition), environmental (temperature, pH) and anthropogenic (fishery) risk-factors can affect the susceptibility of the fish to develop skin ulcerations. This study has also raised further questions, which can be worthwhile to be addressed in future research to fully elucidate the multifactorial etiology of skin ulcerations.

Samenvatting

Platvissen (Orde Pleuronectiformes) zijn fascinerende dieren die in de zee, in estuaria en soms zelfs in zoetwater leven. Ze kunnen herkend worden aan hun afgeplat lichaam met hun twee ogen aan éénzelfde kant en hun aangepaste zijdelingse zwembewegingen. In de gastronomische wereld kunnen platvissen wel gesmaakt worden wat hen een economische waarde geeft. Ook in het mariene ecosysteem vervullen ze een belangrijke ecologische rol in het voedselweb als predator- en prooidier.

Het mariene ecosysteem waar platvissen in leven wordt voortdurend beïnvloed door verscheidene menselijke activiteiten en invloeden zoals scheepvaart, visserij, dumpen van baggerzand en uitstoot van broeikasgassen. Deze activiteiten of veranderingen in het ecosysteem kunnen biologische effecten veroorzaken waaronder het ontwikkelen van ziekten zoals huidzweren, letsels waar de epidermis en basale membraan ontbreken (**Hoofdstuk 1**). Al verschillende jaren worden internationale, gestandaardiseerde monitoringscampagnes uitgevoerd die de prevalentie van deze ziekten controleren in de Noordzee en aangrenzende gebieden en op deze manier een uitspraak kunnen doen over de gezondheid van het ecosysteem. Hoewel het voorkomen van huidzweren gecontroleerd wordt tijdens internationale visziekte monitoringcampagnes op zee werden de exacte oorzaak en risicofactoren die betrokken kunnen zijn bij de ontwikkeling van huidzweren nog niet vastgesteld. Het lijkt misschien eenvoudig om een oorzaak te vinden voor een letsel maar dit is helemaal niet het geval. Zowel vissen als het mariene ecosysteem zijn zeer complex, bovendien zijn beiden heel nauw verbonden met elkaar. Daarenboven is de zee zeer variabel zowel in tijd als in ruimte en speelt dit alles zich af uit het zicht, verborgen onder het wateroppervlak (**Hoofdstuk 1**).

Tijdens de monitoringscampagne in het Belgisch deel van de Noordzee werd een plotse en onverklaarbare stijging in de prevalentie van huidzweren waargenomen in de populatie van schar (*Limanda limanda)* tussen 2011 en 2014. Dit bracht een grondig onderzoek op gang naar de etiologie of oorzaak van deze ziekte.

Een algemeen kader dat stelt dat ziekten in de meeste gevallen gelinkt kunnen worden aan een onbalans tussen pathogenen, vis-gerelateerde factoren en omgevingsparameters, werd reeds beschreven en wordt uitgebreid besproken in **Hoofdstuk 1**. Dit kader werd gebruikt als leidraad doorheen dit onderzoek met als doelstelling om de wetenschappelijke kennis omtrent de oorzaak van huidzweren bij schar uit het Belgisch deel van de Noordzee te vergroten (**Hoofdstuk 2**). Om dit doel te bereiken werd een geïntegreerde, allesomvattende en multidisciplinaire aanpak gevolgd waarbij resultaten van monitoring campagnes op zee gecombineerd werden met laboratoriumexperimenten om pathogenen (**Hoofdstuk 4, 5, 6 en 7**), vis-gerelateerde factoren (**Hoofdstuk 7**) en omgevingsparameters (**Hoofdstuk 7**) te bestuderen.

DEEL I: Verkennende studie

Het onderzoek startte met een eerste verkennende studie in het Belgisch deel van de Noordzee om de letsels te bestuderen en de eerste indicaties te verzamelen van factoren die mogelijks betrokken zijn bij hun ontwikkeling (**Hoofdstuk 3**). Er werden verschillende scharren met huidzweren gevonden en zorgvuldig onderzocht. In deze zweren werden twee bacteriën meermaals geïsoleerd, namelijk *Vibrio tapetis* en *Aeromonas salmonicida*. *V. tapetis* is een gekende pathogeen voor tweekleppigen die 'Brown Ring Disease' veroorzaakt. Hoewel deze bacterie in de laatste jaren af en toe werd teruggevonden bij vissen in gevangenschap, was het de eerste keer dat *V. tapetis* werd gevonden bij een wild-gevangen mariene vissoort. *A. salmonicida* is tevens een gekende vis pathogeen; toch werd in de isolaten die hier verzameld werden een specifieke, nog onbeschreven A-laag gevonden. De A-laag vormt een extra laag aan de buitenkant van de kiem en wordt geacht een belangrijke virulentiefactor te zijn voor de bacterie die deze beschermt tijdens de infectie van het weefsel van de vis.
Deze verkennende studie kan gezien worden als een goed aanknopingspunt voor verder onderzoek naar factoren die betrokken zijn bij de ontwikkeling van huidzweren.

DEEL II: Vis - pathogeen interactie

Om de betrokkenheid van de vernoemde bacteriële soorten in de ontwikkeling van huidzweren bij schar te bevestigen, werd een *in vivo* infectieproef uitgevoerd. Naast het bestuderen van de rol van de bacteriën werd ook de rol van de intacte huidbarrière en het verschil tussen de gepigmenteerde en de niet-gepigmenteerde zijde onderzocht. De vissen werden in contact gebracht met de bacteriën (afzonderlijk) en nadien voor 21 dagen opgevolgd waarbij sterfte, klinische symptomen en de ontwikkelingen van de huidzweren werden bestudeerd. Hierna werden de vissen opgeofferd en bemonsterd.

Vier dagen na infectie met *V. tapetis* (**Hoofdstuk 4**) stierven verschillende vissen. Vissen die met de bacterie geïnfecteerd werden ontwikkelden ergere huidzweren dan de vissen die niet geïnfecteerd werden. Door deze resultaten, veronderstellen we dat *V. tapetis* weldegelijk een rol speelt in de ontwikkeling van deze letsels. Bovendien werden de meeste huidzweren teruggevonden op de plaats waar we de huid beschadigden wat suggereert dat een beschadiging van de huid een belangrijke factor is die bijdraagt aan de ontwikkeling van huidzweren.

Verder onderzoek werd uitgevoerd naar de specificiteit van de *V. tapetis* isolaten die gevonden werden in schar, andere vissoorten en tweekleppigen (**Hoofdstuk 5**). De variabiliteit werd bestudeerd door middel van een *in vivo* virulentie test, genetische (sequenering van het virB4 gen) en eiwitgebaseerde (MALDI-TOF MS) methoden. Helaas bleek geen van de drie methoden geschikt om een onderscheid te maken tussen de isolaten op basis van hun gastheer. Verder werden de drie methoden vergeleken en geëvalueerd in een poging om een methode te vinden om de isolaten te onderscheiden op basis van hun pathogeniciteit voor tweekleppigen of vissen, ongeacht hun afkomst. De eiwit-gebaseerde methode leek in staat om een onderscheid te maken tussen isolaten die pathogeen zijn voor tweekleppigen en degene die dat niet zijn.

Na infectie met *A. salmonicida* (**Hoofdstuk 6**), werd geen sterfte vastgesteld tot 12 dagen na infectie waarna de sterfte gradueel begon. De meeste zweren ontwikkelden in de zone waar de huid verwijderd werd, wat opnieuw wijst op een belangrijke rol van een intacte huid barrière ter bescherming tegen bacteriële invasie en ontwikkeling van huidzweren. *A. salmonicida* lijkt betrokken te zijn in de ontwikkeling van huidzweren bij schar maar wat de exacte rol en pathogenese van deze bacterie is moet nog verder onderzocht worden.

DEEL III: Geïntegreerde studie

Om mogelijke oorzaken van huidzweren te onderzoeken werden tweemaandelijkse monitoringscampagnes uitgevoerd op acht vaste staalnamepunten in het Belgisch deel van de Noordzee (2016-2019). Om dit op een geïntegreerde manier te onderzoeken werd de rol van pathogenen, vis-gerelateerde en omgevingsfactoren onderzocht (**Chapter 7**).

Pathogenen: De rol van *V. tapetis* in de ontwikkeling van huidzweren werd bevestigd door isolatie uit verschillende huidzweren van verschillende vissen gevangen tijdens dit onderzoek. Verder bleek ook *V. alginolyticus* belangrijk. Andere bacteriële genera die werden teruggevonden in een minderheid van de letsels zijn *Bacillus* sp., *Psychrobacter* sp., en *Actinobacter* sp. *Aeromonas* sp. werd ook geïsoleerd uit één letsel maar kon niet tot soort-niveau geïdentificeerd worden

Vis gerelateerde factoren: De meeste vissen hadden één zweer, hoewel we ook vissen vingen met vijf zweren. Huidzweren bleken voornamelijk aanwezig op de niet-gepigmenteerde zijde. Grotere vissen hadden een grotere kans om een huidzweer te ontwikkelen. Een lagere conditie kon geassocieerd worden met de aanwezigheid van een ulceratie. Het geslacht van de vis leek geen invloed te hebben.

Omgevingsgerelateerde factoren: Uit deze studie blijkt dat de zeewater temperatuur en pH een positief verband tonen met de aanwezigheid van huidzweren dat wil zeggen als de temperatuur en pH stijgen, meer vissen huidzweren kunnen ontwikkelen. Verder was ook de visserij intensiteit positief gerelateerd met de huidzweren.

Belangrijk om te onthouden is dat deze veronderstellingen enkel gebaseerd zijn op observaties, hierdoor kunnen geen oorzakelijke verbanden vastgelegd worden. In de reeds beschreven studie werd de rol van V. tapetis en A. salmonicida in huidzweren reeds aangetoond. Er zullen experimenten uitgevoerd moeten worden om oorzakelijke verbanden te kunnen bewijzen bij de andere factoren die een correlatie vertoonden met aanwezigheid van huidzweren.

Zoals duidelijk aangetoond werd in de vernoemde onderzoeken kunnen verschillende factoren interageren met elkaar en op die manier een effect uitoefenen dat kan resulteren in het ontwikkeling van huidzweren. Om pathogene agentia, vis-gerelateerde factoren en omgevingsfactoren te kunnen integreren, werd een innovatief *in vitro* model ontwikkeld. Een 'tweekamer huid explant model' werd ontworpen waarbij rekening gehouden wordt met het verschil in saliniteit tussen de omgeving buiten en binnenin een vis (**Hoofdstuk 8**). Na één dag in het model vertoonde de huid geen grote verschillen met een controle huid. Er werden minimale histologische verschillen waargenomen in de structuur van de huid, het aantal cellagen, het aantal slijmbekercellen en het aantal eosinofiele granulocyten. Bovendien vertoonde de huid geen proliferatie of apoptose. Enkel een kleine stijging in

epidermale dikte werd geobserveerd. Dit alles wijst erop dat het ontwikkelde model een allesomvattend en waardevol *in vitro* alternatief kan zijn met mogelijke toepassingen in het onderzoek naar de oorzaken van huidzweren.

Dit onderzoek was toegespitst op verschillende afzonderlijke aspecten van huidzweren in schar uit het Belgisch deel van de Noordzee, waarbij pathogene agentia, visgerelateerde factoren en omgevingsfactoren afzonderlijk werden bestudeerd. We eindigen deze studie met een bespreking van een aantal algemene punten en proberen verschillende resultaten te combineren. In de algemene discussie (**Hoofdstuk 9**) wordt een overkoepelende analyse voorzien van de onderzoeksmethodologie en de resultaten. Verder halen we nog aan wat de toekomst kan brengen voor het onderzoek. De resultaten uit deze studie worden ook gebruikt voor een terugblik op de plotse stijging in zweer prevalentie waargenomen tussen 2011 en 2014 waarbij een geografische herverdeling van de pathogene *V. tapetis* en een verandering in visserij techniek en/of intensiteit in het Belgisch deel van de Noordzee worden besproken als mogelijk aanleidingen voor de waargenomen stijging.

Ter conclusie (**Hoofdstuk 9**), door de combinatie van verschillende onderzoeksmethoden en het vergelijken van hun resultaten, kunnen we concluderen dat huidzweren inderdaad een multifactoriële etiologie hebben. Beschadiging van de huid is een belangrijke factor in de ontwikkeling van huidzweren en deze letsels kunnen vervolgens gekoloniseerd worden door pathogenen zoals *V. tapetis* en *A. salmonicida*. Verschillende vis-gerelateerde (lengte, conditie), omgevings-gerelateerde (temperatuur en pH) en antropogene (visserij-intensiteit) risicofactoren kunnen de gevoeligheid van de vis voor het ontwikkelen van deze letsels beïnvloeden. Deze studie belicht ook een aantal nieuwe vragen voor toekomstig onderzoek om de etiologie van huidzweren verder te ontrafelen.

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Schol ;)

Maaike

Curriculum vitae

Maaike Vercauteren werd geboren op 5 maart 1991 te Sint-Niklaas. Na het vervolledigen van het secundair onderwijs, richting Wetenschappen-Wiskunde in de Broederschool Humaniora te Sint-Niklaas, startte ze in 2009 met de opleiding Biologie aan de Universiteit Gent. In 2014 behaalde ze het diploma 'Master of Science in Biology' met grootste onderscheiding. Tijdens haar opleiding behaalde ze ook het certificaat proefleider. Na deze opleiding volgde ze succesvol de specifieke leraren opleiding aan de Universiteit Gent waar ze slaagde met grote onderscheiding in 2015.

In 2015 begon ze vervolgens als wetenschappelijk medewerker onder begeleiding van Prof. dr. Annemie Decostere, Prof. dr. Koen Chiers en Ir. Lisa Devriese. Ze werkte aan een project rond de oorzaak van zweren bij platvissen, een project gefinancierd door het toenmalige Europees Visserijfonds. Gedurende dit project werd ook een FWO-doctoraatsbeurs aangevraagd voor het verderzetten van dit onderzoek. Na een geslaagde verdediging begon Maaike in 2016 aan haar doctoraatsonderzoek aan de vakgroep Pathologie, Bacteriologie en Pluimveeziekten in nauwe samenwerking met het Instituut voor Landbouw-, Visserij- en Voedingsonderzoek (ILVO) en het Vlaams Instituut voor de Zee (VLIZ). Haar onderzoek was gericht op het in kaart brengen van de oorzaak van zweren bij platvissen in het Belgisch deel van de Noordzee en het onderzoeken van mogelijke risicofactoren die de kans op de ontwikkeling van dergelijke letsels kunnen verhogen. Verder werkte ze tijdens haar mandaat intensief samen met het 'Institut Universitaire Européen de la Mer' in Brest in Frankrijk. Tijdens deze samenwerking kreeg ze de mogelijkheid om twee weken mee te volgen in hun onderzoeksinstituut. Bovendien kwam ook een doctoraatsstudent van Frankrijk twee weken naar de Faculteit Diergeneeskunde om mee te lopen met het onderzoek van Maaike. Bovendien werd de VLIZ Communication Award uitgereikt voor het ondersteunen van de communicatie rond de onderzoeksresultaten. In kader hiervan werd een website opgestart waar professionele en recreatieve vissers waarnemingen van zieke vissen konden melden (www.platvisziekten.be).

Maaike volgde verschillende cursussen uit de opleiding Doctoral School van de Universiteit Gent. Ze was promotor van zeven masterproefstudenten en gaf presentaties op verschillende internationale en nationale congressen en symposia. Ze is auteur en co-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften en trad tevens op als reviewer voor verschillende publicaties.

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VLIZ Communication award 2018 – Skin ulcerations in wild flatfish: A mystery to be resolved. www.platvisziekten.be

Contributions on scientific events

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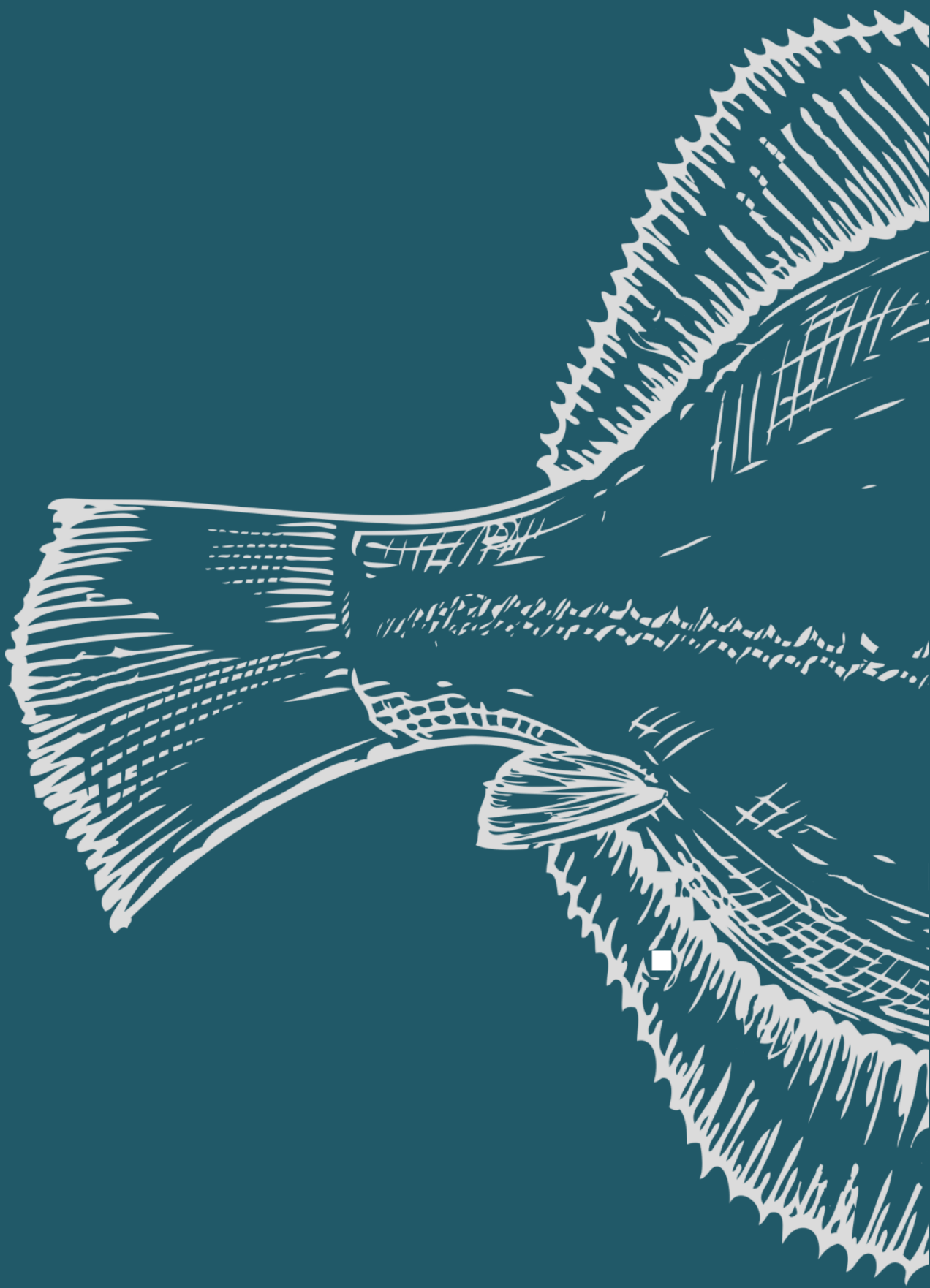

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