Pulse trawl fishing: The effects on dab (*Limanda limanda*)

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Summary

Wild-caught dab (*Limanda limanda*) were exposed to two different electrical pulse stimuli both commercially applied in Dutch flatfish trawls. The experiment was conducted at the IMARES-Yerseke facility, where dab were tested in two groups of 51. The first group was exposed to a Delmeco pulse treatment and the second to a HFK pulse exposure, while a third group was used as "control" group similarly without being exposed electrically. The pulse treatment was given in the closest range of a conductor with a dose extending the commercially applied practice. The fish were kept in observation for five days after the treatment, after they were transferred alive to the Central Veterinary Institute (CVI), Lelystad and analysed for external and internal lesions, possibly attributable to pulse exposure. In case of lesions attributable to infections, bacteriological tests were conducted. During the research samples of fish were taken as references for the condition of the fish directly after the catch and directly after the treatment and analysed likewise.

Of the 102 electrically exposed dab, two fishes died after the treatment with unclear relation to the treatment. Dissection results showed that external and internal anomalies occurred in all groups (including the control group), with no clear differences between the exposed categories. Approximately 12 % of the fish contained a *Glugea* infection in their gut, and only in two cases, a bacterial disease was found. In the control group a fish gut contained *Vibro fortis* and in the skin lesion of a HFK exposed fish a primary fish disease *Vibrio anguillarum* was found.

We conclude, that lesions primarily related to pulse exposure were neither observed in the fish analysed directly after the treatment, nor in the fish that were kept in observation for a period of five days after the treatment.

1   Introduction

1.1   Aim of the research

Problem leading to pulse trawling
Pulse trawling is seen as an alternative to beam trawling with heavy tickler chains with advantages in terms of operational costs (fuel) and ecosystem effects (fewer discards). Doubt has been raised about long-term effects on species coming into contact with these gears, e.g. injuries and skin deformations in common dab (*Limanda, limanda* L.). Since the start of pulse gear trawling, the appearance of ulcers in fish were suggested in the media as a negative side-effect of pulse gears and became a debate in European fishing communities.

The aim of this research is to investigate whether electric stimuli practiced in pulse gear could cause injuries in dab, and enhance the development of diseases, such as ulceration. The report gives results for a number of samples analysed.

1.2   Diseases in common dab, a flatfish

Regarding common dab, there are only a few reviews on the occurrence of diseases in the North Sea, Kattegat, and German Bight (Van Banning, 1987; Möller and Anders, 1992), but this phenomenon was already reported in 1905 and 1925 (Mellergaard and Nielsen, 1997). The most common diseases in dab are: 1) lymphocystis, 2) epidermal hyperplasia/papilloma, 3) infectious ulcerative diseases, like vibriosis, and 4) infection with the parasite *Glugea stephani* (Van Banning, 1987; Möller and Anders, 1992; Khan, 2004, Watermann et al., 1987).

1.2.1   Lymphocystis disease

Lymphocystis disease is a chronic disease of freshwater and marine fish all over the world. It is recognized by irregular cauliflower-like lesions on the body surface, including mouth region, fin and tail region. It is a viral infection, which rarely causes mortality. Infection causes transformation and hypertrophy (approximately 1000x) of dermis cells, forming grossly visible lymphocystis nodules, as well as transformation and hypertrophy in cells of the connective tissues of various internal organs (Essbauer and Ahne, 2001). The lesions are sometimes called lymphocystis tumor cells, which are grossly evident as white spots on the skin and fins of infected fish (Harikrishnan et al., 2010). These lesions proliferate as epithelial tumors in some cases (Samalecos, 1986). The genus Lymphocystivirus has at least two viral species (Essbauer & Ahne, 2001): Lymphocystis disease virus 1 (LCDV-1) and Lymphocystis disease virus 2 (LCDV-2). LCDV-1 infects European flounder (*Platichthys flesus* L.) and European plaice (*Pleuronectes platessa* L.). LCDV-2 infects the common dab (Figure 1a, Appendix C).

1.2.2   Epidermal hyperplasia/papilloma

Epidermal hyperplasia/papilloma is a disease, in which fish, such as dab show whitish opaque swellings of the epidermis on trunk and fins, of a few millimetres high and up to 20 mm in diameter. Single or multiple lesions can be found on the same fish. The upper side is more affected than the blind side. The disease is possibly virus induced. Adenovirus-like particles have been observed in both epidermal hyperplasia and epidermal papilloma (Watermann et al., 1987), Figure 1b.

1.2.3   Ulcerative diseases

The occurrence of ulcers in wild fish is not a recent phenomenon and reported as early as 1905 and 1925 (Mellergaard and Nielsen, 1997). In the early 1970s indications that these diseases were linked to environmental stress became generally accepted (Wedemeyer, 1970; Snieszko 1974). Studies in European waters in the 1980s showed that, eel (), cod (*Gadus morhua* L.), saithe () and dab were heavily affected by ulcerative disease (Möller and Anders, 1986). Ulcers in marine fish may be caused by various bacteria, among which *Vibrio* species (Ortigosa et al., 1989; Austin & Austin, 2012). Often, secondary infections may be the case, with a primary cause related to a number of environmental

conditions, such as water quality changes, low physical condition, and an injured skin. There are a few primary pathogenic bacteria for fish, like *Vibrio anguillarum*, which may cause serious disease and mortality (Austin & Austin, 2012).

1.2.4 *Glugea stephani infections*

The microsporidan *Glugea stephani* (Khan, 2004) is a common gut parasite of dab and other pleuronectid flatfishes. Fish get infected at water temperatures above 14 °C. Vectors, like brine shrimp (*Artemia salina*) and amphipods (*Corophium spinicorne*) may transmit the parasite to fish, but fish can be as well infected by direct ingestion of spores by the host. The parasite is potentially lethal to young pleuronectid flatfishes when heavy infections involve the entire intestine and reduce the capacity to absorb nutrients. Under these circumstances, starvation is probably the direct or indirect cause of death (Olson, 1976).

1.2.5 *Dab diseases in the North Sea*

In North Sea dab, very high prevalence of ulcers was repeatedly found in samples around the Doggerbank area. In the summer of 1980 a contribution of 34 % of ulcers was found.
Between 1983 and 1993 fishing surveys were conducted in the mainframe of the NSTF (North Sea Task Force) in four areas in the German Bight and two areas along the west coast of Denmark (Mellergaard & Nielsen, 1997). The presence of lymphocystis, epidermal papilloma and skin ulcers in common dab were studied using a total sample of 53302 dab. Significant similarities in temporal trends of diseases were observed in samples coming from the German and Danish waters. A similar trend was not observed in samples from the Skagerrak. In 1988 the prevalence of lymphocystis, epidermal papilloma, increased significantly without a clear background. The highest prevalence of lymphocystis was observed in 1988 along the most off-shore area of Danish west coast with a peak value of 14.9 %. In that period epidermal papilloma peaked at 9.4 % in catches from the German Bight. The disease pattern observed in the Skagerrak samples differed significantly from samples of the other areas with skin ulcers as the most prevalent disease. The occurrence of skin ulcers in the Skagerrak did not reveal any specific temporal trend and had a peak of 4.4 %.

1.3 Electric pulse gear development

In the 1960s heavy beam trawls were introduced and quickly outcompeted the otter trawl as the mostly applied gear in the fishery for sole (*Solea solea* L.) and plaice (*Pleuronectes platessa L.*) in the North Sea. In particular the increase of fishing capacity of the trawl expressed in the number of tickler chains and the increase of the towing speed contributed to the catch efficiency (Daan, 1997; Rijnsdorp et al., 2008). The downside of this development was an increase in fuel cost, bycatch of undersized fish and impact on the benthic ecosystem (de Groot, 1984; Jennings and Kaiser, 1998; Poos et al., 2013).

Experiments to reduce fuel consumption by applying an experimental electric stimulus in shrimp and flatfish trawls started in the sixties (de Groot & Boonstra 1970). Since 1992, the technique has evolved (van Stralen, 2005; Polet at al., 2005a, 2005b) with a first commercial application of pulse gear on board the beam trawler UK153 in 2004. By the end of 2014, eighty-four commercial beam trawlers have a temporary licence for fishing in the North Sea with pulse gear, the majority for sole and plaice and three for brown shrimps *Crangon crangon*. All vessels operate under a temporary licence since Council Regulation EC Reg. 850/1998 prohibited the use of electrical stimulation in fishing gear (EU, 1998).

The history and development of the current pulse fishing technique has been reviewed by Soetaert et al. 2013 and van Marlen et al., 2014. Van Marlen et al. (2014) compared the catches of two pulse trawlers fishing with two differing systems against a vessel with traditionally rigged gears with tickler chains. The results showed that pulse trawl catches contained less undersized fish (discards) and benthic invertebrates, while the catch of marketable sole and plaice per unit of area fished were not statistically different from the traditional beam trawl. As the overall weight of pulse trawls is much lower and the gears are towed at a lower speed (e.g. 5 instead of 6.5 knots) causing a lower gear drag, the fuel consumption, fuel costs and associated CO_2 emissions are reduced substantially.

Research on the effects of pulse fishing on marine biota has been required by the EU and ICES in support of temporally licences given to vessels operating these gears. To address the international concern on the effects of pulse stimuli tank experiments were conducted on cod (de Haan et al., 2009a and 2011), dogfish (*Scyliorhinus canicula* L.) (de Haan et al., 2009b) and six species of benthic invertebrates (van Marlen et al. 2009). The results of these studies showed that cod exposed in the close range of the conductors could sustain severe vertebral injuries, and effects on the tested marine fauna were minimal, with no significant differences in survival.

1.4   Effects of fish to electric stimuli

According to Whitney and Pierce (1957), Halsband (1967), and Emery (1984), Van Harreveld, (1938) and Kolz, (1993), the response of fish to electric field exposure depends on the species, size, muscular mass, shape, condition, surface area, epidermis and possibly even the size of the scales, but even when species have the same nervous system they could respond differently (Soetaert et al., 2013). Most of these studies refer to stunning experiments in freshwater experiments. In saltwater current intensity through the fish body has a lower contribution and depends on the electric properties of the morphology of the fish, which is species-dependent with variable quantities for conductivity, capacitance, and impedance (Sternin et al., 1972, 1976; Sharber et al., 1995). Three basic types of reactions were found: the minimum response, electrotaxis and electronarcosis (Mck Bary, 1956). Injuries in fish exposed to electric stimulus mainly refer to vertebral injuries. Sharber and Carothers (1988) found vertebral injuries in 50 % of the 209 trout (*Salmo gairdneri*). Salmonid species are reported as highly sensitive to electric stimuli with contributions of spinal injuries and haemorrhages in 50 % of the dissected fish. Wild-caught Atlantic herring (*Clupea harengus*) exposed to a 50 Hz AC sinusoidal stimulus suffered vertebral injuries and haemorrhages in 60 % of the 260 exposed fish (Nordgreen et al., 2008). These studies refer to settings with very high duty cycles >30 %, while duty cycles applied in pulse trawls are 2 %. Duty cycle D (%) is the ratio of the "active" duration T_{on} (s) of the pulse and the pulse cycle T_{cycle} (s) and is given by $D = \frac{T_{on}}{T_{cycle}} * 100$. A description of the pulse technique and the exposure of the seabed and the effects to cod is reported by de Haan et al., 2015 (in prep).

1.4.1   Observations of ulcers in dab since the introduction of pulse gear

Monitoring programmes of commercial catches along the Belgian coast showed the prevalence of ulcers in dab was stable around 1.6 % for many years, but this number increased to 3 % in 2011 and 6 % in 2012 with no explanation for the sudden increase (*pers. communication* H. Polet ILVO Fishery Ostend, Belgium, Fishing news International September 2012). As pulse exposure was suggested to cause direct skin lesions in the media, the Belgian fisheries Institute ILVO conducted a number of unreported short tests with dab exposed on top of a Delmeco conductor. They did not observe a direct effect of the pulses, like skin lesions (*pers.communication* H. Polet). Samples of dab (7) and plaice (13) showing ulcers were analysed at the pathology division of the University of Ghent, Belgium. The diagnosis was a macroscopic and histologic confirmation of ulcerative disease (Chiers and Decostere, 2012). However, given the developed stage of the infection, a primary cause like pulse exposure could not be determined. The origin was suggested to be a mixture of hypoxic, environmental conditions, and viral and bacterial pathogens, with possibly stress and trauma.
On behalf of the Dutch Ministry of Economic Affairs the Fish, Crustacean and Shellfish Disease Laboratory of Central Veterinary Institute (CVI), part of Wageningen UR, at Lelystad analysed a sample of 4 dab (Ref 12010586-9) with *Glugea* and ulcers (Figure 2) taken from a commercial pulse trawl catch in May 2012. The diagnosis showed ulcers, which seemed to have a mechanical cause, and *Glugea,* but a relationship with pulse gear fishing could not be confirmed nor be excluded.
As knowledge on the direct effects of pulse exposure was lacking, we conducted a controlled experiment and exposed wild-caught dab to pulses and analysed the direct and post-response observations, in particular external and internal lesions.

2 Materials and Methods

2.1 Pulse experiment

On 25 April 2014, individual wild-caught dab were exposed to two different pulse stimuli in groups of 51 specimen each, while a third group of 50 dab was treated as "control" reference, without being electrically exposed. The experiment was conducted at the IMARES laboratory in Yerseke. The response to the exposure was filmed underwater at the moment of the treatment and the post-response observed over a period of five days after the treatment. The overview of the exposures and samples are listed in Table 2. In the period between the catch of the fish and the pulse treatment, samples of five dab were taken shortly after the catch ("ref catch") and directly after the pulse treatment three samples were taken per tested category, each reflecting the condition of the fish directly after execution of the particular treatment "test-post" (Table 2). After the observed period the final sample were transported alive and analysed the same day ("test-end").

2.1.1 Origin of the experimental dab, procedures and holding conditions

On 8 April 2014 the experiment was approved by the Dutch animal welfare committee under number 2014032 to collect a number of 289 dab. On 16 April 2014 the fish were caught from the North Sea by using the shrimp beam trawls of the Dutch trawler OD2. Gear parts that could cause injury, such as the interior sieve panels and tickler chains connected to the footrope, were taken off. A number of 13 hauls of 5 to 15 minutes were carried out at a towing speed of 2-4 knots to catch the approved number. The fished location was off the Dutch coast near Stellendam harbour, locally known as "Slijkgat". Local water depth on the fished area was around 4-10 m. Catches were released into a selection tank from where the fish were transferred and stabilised in three survival tanks positioned on the ship's deck. These tanks were refreshed with seawater pumped by the ship's facility. From the catches 57 dab with strong lesions and anomalies were discarded, after they were photographed and length measured as reference to the condition of the fish and the discarded total. Of the selected 289 dab, 36 dab were randomly photographed and length measured. The length of the collected fish ranged between 15 and 25 cm. On arrival in Stellendam harbour, the fish tanks were directly transferred to the IMARES laboratory in Yerseke. During the transport the tanks were aerated with oxygen.

2.1.2 Stabilising of the collected fish

On arrival at the IMARES laboratory the fish were randomly divided over three main holding tanks positioned on the outer yard of the laboratory (Figure 3). The day after, a total of 8 dead fishes were taken out. The turbidity of the pumped seawater and the unknown condition of fish sheltered in the sand layer obscured accurate numbers of mortality. Hence priority was given to undisturbed recovery while the response to feeding cycles was taken as broad measure of the condition of the fish.

2.1.3 Holding facility and initial tests

Three circular tanks of 1.76 m diameter were used as main storage to stabilise the fish in the period between catch and treatment. The tanks were connected in parallel to the main seawater inlet from the Oosterschelde and set to a water level of 0.94 m. To keep the environment for the fish as natural as possible Oosterschelde water was not treated and directly pumped to the fish tanks. A flow rate of 1750 l/h was chosen in relation to the relatively high air temperature in the daytime for this time of the year (25°C). Under flow condition the seawater temperature in the main holding tanks could be limited to 13 °C maximum (Figure 4). A substrate of 3.5 cm calibrated sand (grain 0.2 to 0.5 mm), was added to provide shelter for the fish and to keep the circumstances as natural as possible. Tanks were covered with flexible netting to avoid predation by birds and to prevent fish from jumping out.

2.1.4 Holding tanks for treated fish

Nine tanks (three per treatment category) of 1.2 x 1.0 m were used to hold and observe post effects after treatment. As the positioning of the nine tanks (Figure 3) could have a side-effect on the condition of the fish, the division of treatment categories was randomly selected by drawing lots. The seawater level in the nine tanks was set to 0.5 m. Tanks were connected in parallel to the main seawater inlet with a refresh rate between 750 and 1275 l/h, depending on the position of each tank relative to the main sea water inlet. Seawater temperature in three of the measured tanks was 15 °C at maximum (Figure 5).

2.1.5 Feeding regime

Fish in the holding tanks were set to a daily regime of 20-70 grams of life ragworm (*Nereis virens* L.) per day per tank for the first 5 days of recovery, which was increased to 200-300 grams/day. Two days before the start of the treatment feeding was interrupted and continued directly after completion of the treatment. After the treatment the exposed fish were set to a daily feeding regime of 100 grams/fish/day/tank. Based on a requirement of 2.4 % dry food per 100 grams fish weight and 40 % dry food weight in ragworm, each holding tank containing 17 dab was set to a feeding regime of 100 grams ragworm according the equation $F_w = dab_n * 100 * 2.4/100 * 100/40$).

2.1.6 Equipment used to monitor seawater condition in the holding tanks

As reference to the condition of the fish before and after the treatment seawater temperature, conductivity, pH and oxygen were measured daily using the equipment listed in Table 3a. On the day of the treatment seawater temperature and conductivity in the exposure tank were measured using a WTW Cond 3110 hand-held instrument. The results listed in Table 3b show that these conditions did not vary over the day and were comparable for both pulse treatments.

2.2 Experimental treatment

Fish were transferred in groups of 75 dab to a transfer tank (Figure 1) from where each single fish was transferred to the exposure tank after the overall length was measured. Each fish was positioned in close range of a conductor by using a net of small mesh flexible netting. The fish could freely swim in the netting and the orientation of the fish relative to the conductor was left random to avoid additional stress. The treatment was given once a fish positioned itself on top of the conductor with most of its body at the inner part of the conductor. The selection of an electric or a "control" treatment was random and decided by drawing lots. The electric treatment consisted of a burst of pulses of 1 s pulses mimicking the passage of the length of an electrode, close to the exposure time of 1.16 s estimated for a towing speed of 2.57 m.s-1 (5 knots) and the conductive length of 3 m. The response of each fish was scored and filmed underwater using a black/white bullet camera type Sony 1/3 inch 0.05 lux (Figure 6). The video signals were recorded using a Sony GVD-1000 video recorder and stored on digital DV cassettes. All video images were stored on hard disc in MPEG 4 format. The sequence of treatment of the three tested sub-samples ("test-post") was Delmeco, HFK and "Control" and was executed prior to the treatment of main groups. After completion the treatment was continued with the HFK, followed by the Delmeco stimulus, both treatments randomly altered by drawing lots with dab tested as "Control". Given this schedule, seawater condition as an effect of the tidal change, light and air temperature differed per treatment, although the deviations sampled over the day were minor (Table 3b). The treated fish were distributed over nine holding tanks, with three per category. The selection of a tank was randomly taken by drawing lots to a maximum of 17 fishes per tank, resulting in 51 dab per treatment category.

2.3 Pulse characteristics and equipment

The pulse characteristics are summarised in the overview of Table 4 and listed against the settings of other pulse exposure effect studies. The field strength were measured opposite the centre of the conductor at the level of the spinal cord of the fish.

2.3.1 Delmeco pulse stimulus

The bipolar Delmeco pulse stimulus (TX68) is used in commercial practice and has a symmetrical delay between the alternating pulse parts (Figure 7). The stimulus comprised a 254 µs pulse width, a pulse cycle of 40 Hz and a 12.6 ms bipolar interval (Figure 7). The voltage across the conductors was set at maximum amplitude (64 $V^{0\ to\ peak}$) with a supplied current of 42 A^{0-peak} (Table 4). The pulse hardware was equivalent to all other pulse experiments and included a power supply and pulse generator connected to an electrode section of two conductor pairs (180 x 28 mm) separated by an isolated section of 0.6 m all parts copies of commercially applied materials. In series with the output an inductor was used to achieve the exponential rising edge of the pulse (Figure 7). The electrodes were set at a distance of 0.325 m identical to the set-up applied in other pulse effects studies done by IMARES on marine biota (de Haan et al., 2009a and b and 2011 and van Marlen et al., 2009).

2.3.2 HFK pulse equipment

The HFK pulse stimulus varies in practice between a bipolar pulse with a symmetrical pulse shape such as the Delmeco stimulus, or with a minor bipolar interval (Figure 8). To test a second pulse type the HFK-stimulus was generated with a minor interval (40 µs) between the alternating pulse parts. Secondly, this pulse type was also experienced in the study on the effects on cod (de Haan et al., 2011). The pulse simulation hardware and electrodes was identical to the hardware used on the experiments on cod (de Haan et al., 2011). The power supply consisted of the Delmeco adjustable DC power source and the HFK MOS-FET H-Bridge discharge circuitry including a buffer capacitor of 22400 µF (2x4400 and 2x6800). A damping resistor of 42 Ω was connected in parallel to discharge the capacitor bridge on de-activation. The electrodes contained a single pair of conductors (125 x 27 mm) and were set to 0.325 m distance.

2.3.3 Pulse measurements and equipment

Field strength (V/m) was measured 5 mm above the bottom and 55 mm opposite the centre of the conductor (Table 4). Field strength opposite the centre of the conductor was 288 $V.m^{-1}$ and 240 $V.m^{-1}$ (Delmeco) and the regression towards the conductor ends fitted a polynomial function (HFK: y = -0.0138x2 + 0.0364x + 265.3, DEL y = -0.0102x2 + 0.3158x + 252.16). Field strength was not influenced by the netting used to position the fish. Field strength was measured using a probe with two isolated poles with the sensing heads facing in opposite direction at a fixed distance of 25 mm. For each measurement session the probe distance was measured. Pulse parameters were measured by using a 200 MHz LeCroy WaveSurfer 24XS oscilloscope and a LeCroy differential AP305 high voltage probe connected at the conductors. An inductive clamped current probe (type LeCroy AP015 30 A, SN 7968) to measure the input current of the supplied energy. This probe operates from DC to 50 MHz and was calibrated and degaussed shortly before the start of the measurements. The voltage related to field strength was measured using a probe with two isolated poles with the sensing heads positioned opposite each other at a fixed distance of 25 mm. The signal was measured using a low-voltage differential AP031 70V probe. All voltage measurements in this paper refer to the peak voltage of the positive amplitude of the bipolar pulse relative to the zero. Samples of measurement results were stored as JPEG images on hard disc of the oscilloscope.

2.4   Analysis and dissection procedures

On 30 April 2014, five days after the treatment all remaining fish were transported alive to the CVI-laboratory. The fish were conditioned in plastic bags with seawater from the holding tanks. Oxygen was added to each bag to make sure the oxygen concentration in the water was sufficient during transport and stored in an isolated transport tank. Each sample was labelled with a CVI-ID number and administrated with the standard CVI-analysis document (Table 2). The transport of any sample was scheduled to execute the analysis the day of arrival. None of the fish died during transport. The number of analysts involved was adapted to conduct all routines with groups analysed in parallel within the available time.

The fish were euthanized on arrival by an overdose of 2-phenoxy ethanol and length and weight were measured, after which clinical signs of disease and deformities were scored.

The nine samples were analysed systematically and partly in parallel, so that there was no time effect which could affect the result.
The arrived numbers of the three treated categories and sub-groups (sub number 1-3) were analysed:
- REF: 17 + 17 + 16
- DEL: 17 + 17 + 17
- HFK: 17 + 17 + 15 (+1 dead dab)

2.4.1   Summary of the CVI analysis and methods and equipment used

1. Pictures were taken of single fish and in groups of dorsal and ventral sides;
2. Each fish was checked for clinical signs, including internal and skin lesions;
3. Of a maximum of 5 fishes per subgroup (17) blood samples were taken and checked for blood parasites;
4. In case of ulcers, a maximum of 5 fishes per subgroup were sampled for bacteriology and parasites;
5. Internal organs were visually checked for abnormalities, and in case of ulcers or in case of a congested/enlarged spleen samples from the liver and kidney were taken for bacteriology;
6. For each fish findings were scored on a necropsy form.

A detailed description of the CVI-analysis procedures and methods is given in Appendix A.

2.5   Statistical analysis

The type of injuries sorted per group was tested against the likelihood of a pulse related background. For this purpose four types of injuries were sorted in external and internal anomalies according to the definition below:

Ext haemorrhages:	skin wounds with unknown background (pulse exposure, mechanical)
Int lesions:	Internal lesions other than parasites, like *Glugea*
Int. haemorrhages:	Internal bleedings spec. not excluding pulse effect
Ext lesion:	External anomalies other than haemorrhages, like bacteria *Vibrio fortis*, eroded tail.

In addition to the pulse related background, fish biological features, such as length, weight and prevalence of internal diseases other than wounds (Internal parasites ≈ "Int paras") were also tested for the association with 4 categories of injuries, defined above.

There were two groups with different sample size and different background to be analysed. The "test-post" group with 5 dab, sent immediately after treatment to the CVI-laboratory, was too small in sample size for any statistical inference. For this small group only descriptive statistics were applied.

The "test-end" group with sufficient sample size was analysed using GLMM (Generalised Linear Mixed Model) method with a binomial distribution to model the probability of injury (scored 0 if absent and 1 if present) as a function of stimulus type, fish length, weight and interval parasite status. The coefficients of the model were then estimated using a maximum likelihood estimation method (MLE). We started with a full model including all explanatory variables, and then repeated the model fitting for all sub-models, which contains sub-sets of the explanatory variables. The optimal set of explanatory variables was selected as the optimal model with the minimum Akaike Information Criterion (AIC).

Once the optimal model was found, 1) the p-value of each explanatory variable was determined using an analysis of deviance (chi-squared) test. A p-value of 0.05 (i.e. at 95 % probability) implies a significant relationship of the corresponding explanatory variable with injury. The estimated coefficient indicates the magnitude of the effect. 2) When a non-significant p-value is obtained but the estimated coefficient is large, we then discuss the possibility of failing to detect a true effect (type II error). A detailed description of the test is listed in Appendix B.

3 Results

3.1 Behavioural response of the fish during and after exposure

A common observed response in all dab was a muscular cramp, which immobilised the fish during the exposed duration. The response to both types of pulse stimulus was different. Fish exposed to the Delmeco stimulus contracted the back part of the muscular system to a "U-shape" with tail and head upward, while the muscular response to the HFK pulse looked like a "hoovercraft" take-off lifting the fish body with the side fins downward. On extinguishing of the exposure all fish produced a short strong startle response in upward direction for the Delmeco pulse and in forward direction for the HFK pulse exposure. At the end of the treatment the exposed dab responded well to the food offered for each of the feeding cycles during the observation period of 5 days.

3.2 Injuries

No direct injuries attributable to pulse treatment, such as haemorrhages in the vertebrae section were observed. Skin or other lesions were not observed, neither in the sub-samples sent for reference at the day of exposure, nor in the fish that were analysed 5 days after exposure. Minor haemorrhages were observed, but these were also found in similar numbers and degree in the fish of the "control" group not exposed to the electric stimuli (Table 7, 8 and 9), with the summarised results listed in the overview of Table 1 of main groups tested, excluding the reference group analysed directly after the catch ("ref-catch", 5 fishes, Table 5) and the reference group for the three treatment categories ("test-post", 15 fishes, Table 6).

Table 1 Overview of main groups tested and lesion score per tested category

Treatment	Total nr	Ext haemor	Int haemor	Ext lesions	In paras	Int lesions	Bact. Sampled	Total lesions
Control (REF)	50	8	2	9	7	4	1 (7)	30
Delmeco	51	5	0	7	7	4	0 (5)	23
HFK	50	8	0	8	3	1	1 (7)	20

A single fish exposed to the HFK treatment was found dead a day after, but a relationship with pulse exposure could not be made (HFK group 3, length 25 cm). This fish had a haemorrhagic skin lesion on the belly side before it was treated to the HFK stimulus (Figure 9).
The fish bone remainders of another fish of the HFK group 3 was found in the tank sediment, excluding the opportunity to evaluate the cause of death. *Glugea* infection in the fish gut was found in 12 % of the analysed fish (including the fish of the control group). Of reference groups "ref catch" and "test-post" 10 fishes were tested for bacterial disease, results were all negative. Of the 151 dab sampled as main target groups (Table 1), 19 dab (analysed numbers per group are listed in brackets) were tested for bacterial disease and in two cases with a positive result. In the final control group, a single case with skin lesion contained *Vibrio fortis* bacteria and in the HFK-1 group a dab with a primary bacterial fish disease, *Vibrio anguillarum* was found.

3.3 Statistical analysis

Summary
The statistical test for "test-end" experiments showed no significant association between pulse exposure and any type of injuries observed (p=0.57, 0.11, 0.85 and 0.29 for "Ext haemor", "Int haemor", "Ext lesion" and "Int lesion", respectively). The estimated coefficients of the Delmeco and HFK stimulus (versus REF, "Control") are listed per tested injury category (Appendix B, Table 14, 17 and 21). The small effect sizes, together with a decent sample size exclude the possibility of failing to detect a true large effect (type II error).

Therefore, we conclude that there is no effect of pulse exposure on any type of the injuries observed.
The only statistically significant association was found between "Int para and "Int lesion".
The large positive coefficient (4.09, or odds ratio of exponent (4.09) = 59.74) indicates that fishes with
internal parasites are associated with a higher probability of "Int lesion".

The relationship between stimulus type, internal parasite status, length and injuries for "test-post" experiments are illustrated in Table 10-11 and Figure 14, Appendix B. "Ext haemor" and "Int haemor" were hardly observed in the 15 fishes, despite the stimulus type. Both REF and Delmeco had 3 out 5 fishes with "Ext lesion", however, HFK did not end up with any "Ext lesion". Two out of 5 fishes from Delmeco and HFK had "Int lesion", while REF had only one fish with "Int lesion". All 4 fishes with positive internal parasite ended up with "Int lesion", which is suspicious for an association. Fishes without "Int lesion" seem to have a large length range or higher average length than fishes with "Int lesion". But this observation is not biologically sound, so the finding probably is due to chance. Despite all these descriptions, due to the small sample size, it is not possible to make any solid conclusions on whether there exists an effect of the covariates on the probability of "test-post" injuries.

4 Discussion

4.1 Behavioural response

Dab exposed to the Delmeco stimulus responded with a strong contraction of the dorsal muscle of the fish body (i.e. U-shaped bend as in sole), similar to the cramp observed in Dover sole and plaice (van Stralen, 2005). This U-shape bending and the startle response of the fish after exposure were suggested to contribute in scooping the fish over the footrope (van Stralen, 2005). Dab exposed to the HFK stimulus did not produce a U-shape cramp, indicating the short interval between bipolar pulse parts could be the background of this different behaviour, which could also affect the catch efficiency.

4.2 Injuries and diseases

Skin lesions or haemorrhages attributable to pulse exposure were not observed in the experiments, neither in the sub-group sampled at the day of exposure, nor in the fish examined 5 days after. A similar observation has been made by ILVO of Ostend, Belgium on dab exposed to a burst of 5 s on top of a conductor not developing direct skin lesions. In the media it was suggested that pulse exposure could lead to skin punctures. This suggestion, however, is not supported by the physical conditions of fish surrounded in a high conductive seawater volume. Although the skin of fish could be resistant, a puncturing charge could be developed when the skin is acting as isolating layer and when the charge is not by-passed by channels of conductive fluid. We did not find direct injuries related to pulse treatment that would enhance the development of ulcers, but we cannot exclude any long term effect of pulse exposure making them more prone to infections such as ulcers.

Concerning clinical signs and haemorrhages, various phenomena were seen in the two groups prior to and after the exposure to pulse stimulation, such as small ulcers and mechanical damage, without clear differences between these groups and the control group. Haemorrhages varied from haemorrhages along fins to petechial haemorrhages in the ventral skin. This was also the case for only low percentages of haemorrhages seen internally in the final groups (4 % of negative control dab, 2 % in both pulse exposed groups).

Ulcers: Most tests of bacterial growth (using inoculated agar plates, see Appendix A) gave negative results. Only in the final group of REF dabs, one dab had a skin lesion with *Vibrio fortis*, and in the HFK-1 group, one dab had an internal *Vibrio anguillarum* infection (Figure 10), which is a primary fish disease. As in seawater, many and various *Vibrio* species may be present, among which fish pathogenic *Vibrio* species, like *Vibrio anguillarum* (Ortigosa et al., 1989). These bacteria may seriously affect dab, but it was only a single finding out of 28 bacteriologically sampled dab. Van Banning (1987) found up to 8.3 % of North Sea caught dab with ulcers, in 1982.

Also, in the control groups and the groups treated to pulse exposure, various infection percentages with *Glugea* species, most probably *Glugea stephani* were found in the gut. *Glugea stephani* is a microsporidian parasite, which is common in the intestines of dab in European waters, found only south of 55" latitude in prevalence of 9.1-20.9 % in 1981-1985 in dab of the North Sea (Van Banning, 1987). In *Glugea stephani* infected flounder, the condition factor and blood values were lower and ovarian development inhibited or delayed in Newfoundland (Khan et al, 2004). The dab in this study, however, did not show clinical signs like anorexia.

We concluded that only low percentages of dab per group, prior to, during, and at the end of the experiment showed various internal and/or external lesions, no pathological effects were seen at all related to exposure to pulse in any of the treated groups. In 12 % of the dab a moderate to heavy *Glugea* infection in the gut was seen, and only in a single case, a bacterial fish disease by *Vibrio anguillarum* was found. No lymphocystis disease, or epidermal hyperplasia/papilloma were found.

4.3 Selection of target species and conditions

Initially we proposed to conduct this research on common dab and Dover sole (*Solea solea*) and to study the effects in the winter months on low water temperature conditions (January and February). We limited the tested species to common dab as these targets could be caught near-shore. In wintertime Dover sole traditionally migrate to deeper water more off the coast out of reach for this vessel, extending the time required to collect the fish. Secondly, fishing for sole would also require a heavier gear with tickler chains. All these aspects would affect the health condition of the fish, while dab could be collected without such aggregation in the vicinity of the harbour Stellendam. The treatment of a single target species enabled the use of a second pulse stimulus operational on the Dutch pulse trawls. Getting approval for the experiments by the Dutch Animal Experiment Committee delayed the experiment to the spring of 2014, while we intended to test the fish under low temperature conditions when the fish was thought to be more sensitive to develop ulcers. As a consequence, the seawater temperature in the experiment was much higher (13 to 15 ˚C), which could have improved the physical condition of the fish as indicated by the feeding response of the fish and the low overall mortality during the experiment (8.3 %). Nevertheless the occurrence of direct lesions and haemorrhages was still a primary argument to carry out the experiments under the given condition.

Pulse treatment is not likely to cause direct injuries in flatfish. During the technical development of pulse equipment by Verburg Ltd. Colijnsplaat, the Netherlands in the period 2000-2005 Dover sole was kept in captivity over long periods in tanks and experienced multiple pulse exposure. The fish all survived these tests and negative effects such as skin lesions or other related injuries were not reported (van Stralen, 2005) and were not observed during measurements conducted by IMARES (de Haan pers. Communication).

We compensated the low conductivity of the water (30 PPT versus 34 PPT in other experiments) by increasing the voltage amplitude on the conductor (Table 4) to 30 and 45 % above the commercially applied range and also decreased the conductor distance with from 0.425 m to 0.325 m, so we have confidence that our conclusion that pulse exposure by itself will not cause direct lesions in dab is valid. However, this does not exclude lesions due to mechanical effects.

4.3.1 The occurrence of ulcers and the condition of dab

This present study could not relate a particular injury to the development of ulcers. Ulcers in wild fish stocks have been reported as early as the beginning of the 19th century by Johnstone, 1905 and 1925, (Mellergaard and Nielsen, 1997) and was found off the coastal waters in the central North Sea around the Doggerbank. Of the 289 dab we collected for this experiment anomalies were observed in 20 % of the catch at a location where pulse gear trawls are not used at all. This figure far extends the number of samples taken along the Belgian coast and shows that the occurrence and origin is unclear. When an experiment, as presently reported, would have to start with fish of a basically healthy population of dab, this would not be easy, independent of pulse gear use. In the group of 57 discarded dab a total of 111 anomalies was found, and 5 dab showed none but a pigment failure (Figure 11). The category "lesion" reflects the fish with injuries of a "mechanical" background without visible disease. The fish sorted as "lesion+ref" had a single or multiple disease of which the share of types are shown separately (Figure 12e and f). The diseases mainly refer to bacterial lesions, caused by for instance *Vibrio* spp., and epidermal hyperplasia/papilloma. It must be noted that the determination is based on visual identification from pictures.

4.3.2 Condition of the fish in the holding system

4.3.2.1 Mortality

Due to the turbid seawater and the fish sheltered in the bottom layer the mortality could not be accurately determined within the observation period and became known after the completion of the experiment. The overall mortality, based on the remaining life fish at the end of the experiment was 26 ≈ 9 %. Of this total 30 % (≈ 8 dab) died a day after the catch. The observed mortality over the experimental period concerned two dab, one with unclear cause of death and of the other only the bone

remainders were found in the substrate. This suggests that not only direct mortality after the treatment was minor, but also that the condition of fish could have improved over time.

4.3.2.2   Feeding response

The fish responded well to feeding, in particular directly after exposure. The leftover of live ragworm indicate that the feeding was not too short either.

We analysed the condition of the dab by applying Fulton's condition factor K (Htun-Han, 1978) a measure of an individual fish's health that uses standard weight. Proposed by Fulton in 1904, it assumes that the standard weight of a fish is proportional to the cube of its body length. For all groups analysed in the CVI-laboratory, we found an average K- factor of 0.9 and this matches the seasonal ranges for male and female dab (Htun-Han, 1978).

4.4   Statistical analysis

Due to the small sample size, it is not possible to make solid conclusions on whether there exists an effect of the covariates on the probability of "test-post" injuries. On the other hand, the sample size for the "test-end" experiments was decent (around 50 fishes per stimulus type) and the detected effect sizes were substantially low. Such results imply that it is less likely that there exists a strong effect of stimulus type, but we fail to detect it due to the small sample size (type II error).

The statistical tests (Appendix B) showed that pulse exposure did not bring any physically related injury, but there might be side-effects related to pulse exposure that were not measured in this experiment, such as a chemical related longer-term response.

Dab with internal parasites have a significantly higher probability of also having another internal lesion, which suggests that fishes with internal parasites are sensitive to other internal lesion.

The weight of the fish was excluded as an explanatory variable in the full model, due to the high correlation with length (Pearson correlation coefficient $\rho=0.92$, Figure 13, Appendix B). Including highly correlated explanatory variables (i.e. collinearity between variables) might lead to biased parameter estimates and over fitting of the model.

5 Conclusions

We conclude, that lesions attributable to pulse exposure were not observed in the fish analysed directly after the treatment and in the fish that were kept in observation for a period of five days after the treatment. Only low percentages of dab per group, prior to, during, and at the end of the experiment showed various internal and/or external lesions, no pathological effects were observed attributable to pulse exposure in any of the treated groups. The statistical tests showed that pulse exposure did not bring any physically related injury, but there might be side-effects related to pulse exposure that were not measured in this experiment, such as a chemical related longer-term response.

Acknowledgements

Quality Assurance

IMARES utilises an ISO 9001:2008 certified quality management system (certificate number: 124296-2012-AQ-NLD-RvA). This certificate is valid until 15 December 2015. The organisation has been certified since 27 February 2001. The certification was issued by DNV Certification B.V. Furthermore, the chemical laboratory of the Fish Division has NEN-EN-ISO/IEC 17025:2005 accreditation for test laboratories with number L097. This accreditation is valid until 1th of April 2017 and was first issued on 27 March 1997. Accreditation was granted by the Council for Accreditation. CVI used ISO 9001 accredited tests for this screening.

Table 2. Overview of experimental treatment, timing and CVI sample references.

Date	Event	Reference	Fish (nr)	CVI ID no.
2014-04-16	Collection of fish		289	Total landed number
2014-04-17		Ref catch	5	14005166
2014-04-25	Post treatment	Test-post	5	14005583 REF
			5	14005583 HFK
			5	14005583 DEL
2014-04-30	End-treatment	Test-end	50	14005712 REF In 3 groups 17, 17 and 16
			50	14005712 HFK In 3 groups 17, 17 and 16
			51	14005712 DEL In 3 groups 17 each

Table 3a. Equipment used to measure the condition of the water in the holding tanks

Parameter	Instrument ID	References used
Salinity	Hach HQ14d Portable and CDC401 electrode	Sample standard 1000 µS.cm^{-1}
Temperature	Hanna HI 93510N	Checked on melting ice
Oxygen	Hach HQ40d Portable and LDO101 electrode	100% saturated water sample
pH	Hach HQ40d Portable and PHC101 electrode	Buffer pH 4.00 and 7.00

Table 3b. Conductivity, temperature salinity readings and the discharge conditions for the HFK and Delmeco treatment.

Time	Conductivity (mS/cm)	Temperature (°C)	Salinity (PPT)	HFK$^{0\ to\ peak}$ discharge (V/A)	Delmeco$^{0\ to\ peak}$ discharge (V/A)
08:00	47.6	13.0	30.3	76.4/41	
15:34	47.7	13.8	30.5		61.4/30.4
19:15	47.8	13.5	30.5		

Table 4. Pulse parameters of the pulse stimuli and references to the experiments on cod

Reference	Pulse treatment	Frequency (Hz)	Pulse duration (µs)	Conductor Voltage (V$^{0\ to\ peak}$)	Conductor Current (A$^{0\ to\ peak}$)	Field strength (V/m)	Field strength reference X, Y (mm)
Dab 2014	Delmeco	40	254	61.4	42.3	288	55/5
Dab 2014	HFK	40	300/40[1]	76/70	36	240	55/5
TH10	Delmeco	40	270	51	n.a	158	35, 5
OD17	HFK	45	280	46	n.a	208	35, 5
Cod 2010 "small"	Delmeco	40	254	57	68	259	35/35

[1]delay time between two bipolar pulse parts

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7 Appendix A CVI-analysis methods and results (for STAT-test)

7.1 Procedures of the analysis, methods and equipment used

1. Pictures were taken of all fish. Per 5-10 fish, group pictures were made the dorsal and the ventral side of the fishes;
2. All dab were checked for clinical signs, and these were scored at the necropsy form and sorted in a Excel worksheet: Any lesion, papilloma, or other clinical sign, see form, was noted. Special focus was given to skin lesions;
3. From 5 fish taken randomly from the bucket with fish a blood sample was taken from the caudal vein, and checked for blood parasites in a fresh smear (microscope);
4. In case of ulcers, samples were taken for bacteriology with disposable plastic inoculation needles, onto BHI (Brain Heart Infusion agar with 5% (volume/volume) sheep blood) agar plates, and onto Marine agar plates respectively. Maximum 5 fish per subgroup (17-19) were used for bacteriology;
5. The bellies of all fish were cut open and the internal organs were visually checked for abnormalities;
6. In case of ulcers, or in case of congested/enlarged spleen (pictures were made of the abnormalities and noted) from the fish as sampled under 4), samples from the liver and kidney were taken for bacteriology, on BHI agar only (max. 5 fish per group);
7. From the same 5 fish a skin slime smear was made from head to tail and directly checked for parasites and bacterial load (microscope);
8. From the same 5 fish a fresh gill preparate was made and directly checked for abnormalities, bacterial load and parasites (microscope);
9. From the same 5 fish a smear of the gut contents was made and directly checked for parasites (microscope);
10. Necropsy form was completed per treatment category, and abnormalities were scored;
11. The necropsy form was further completed and signed.

The inoculated agar plates from skin and internal organs were incubated at 22 °C for maximum 7 days. The agar plates were examined daily for bacterial growth. If no bacterial growth was observed, or, if there was multi-bacterial growth, incubation was terminated after 7 days. If there was a predominant or pure culture of bacteria from organs and/or skin lesions, the bacteria were typed biochemically and molecularly identified. The dab were filleted and put on a glass plate with lamp below, to check for haemorrhages and other lesions in the musculature around the vertebral column. Abnormalities were noted and pictures made with label. All dab were opened and the internal organs were macroscopically checked for abnormalities and presence of parasites. In case of ascites or enlarged organs, these were inoculated on BHI agar only (max 5 fish per group). Dab were frozen at -20 ˚C.

7.1.1 Definitions of categories for statistical test

Ext haemorrhages: skin wounds with unknown background (pulse exposure, mechanical)
Int lesions: Internal lesions other than parasites.
Int paras: Prevalence of internal diseases other than wounds, like *Glugea*.
Int. haemorrhages: Internal bleedings spec. not excluding pulse effect.
Ext lesion: External anomalies other than haemorrhages, like bacteria *Vibrio fortis*, eroded tail.
Bacterial sampled cases are marked with "*".

Table 5. Overview of lesions in dab analysed a day after the catch ("Test catch")

Sub-sample "Test catch" 17 April 2014							CVI ID no. 14005166	
Dab no.	Length (cm)	Weight (grms)	Ext haemor	Int haemor	Ext lesions	Int lesions	Int paras	Remarks
1	18.5	n.a.	0	0	1	1	0	Liver with granuloma (*)
2	22.5	n.a.	0	0	0	0	0	
3	15.5	n.a.	0	0	0	0	1	Some *Glugea*
4	24	n.a.	0	0	0	0	0	
5	19	n.a.	0	0	0	0	0	

Table 6. Overview of lesions in dab analysed directly after the treatment ("Test post")

Sub-sample "Test post REF" 25 April 2014							CVI-ID 14005183 REF	
Dab no.	Length (cm)	Weight (grms)	Ext haemor	Int haemor	Ext lesions	Int lesions	Int paras	Remarks
1	22	103.8	0	0	0	0	0	
2	17	41.2	0	0	0	0	0	
3	17	47.7	0	0	1	1	1	Cysts of *Glugea* (*)
4	19	63.7	0	0	1	0	0	Recovered skin lesion (*)
5	23	117.7	0	0	1	0	0	(*)

Sub-sample "Test post DEL" 25 April 2014							CVI-ID 14005183 DEL	
Dab no.	Length (cm)	Weight (grms)	Ext haemor	Int haemor	Ext lesions	Int lesions	Int paras	Remarks
1	26	183.1	0	0	1	0	0	(*)
2	18	56.5	0	0	1	1	1	Cysts of Glugea
3	18	47.6	0	0	0	0	0	Cysts of Glugea (*)
4	20	75.4	0	0	0	0	0	(*)
5	18	51	0	0	1	0	0	(*)

Sub-sample "Test post HFK" 25 April 2014							CVI-ID 14005183 HFK	
Dab no.	Length (cm)	Weight (grms)	Ext haemor	Int haemor	Ext lesions	Int lesions	Int paras	Remarks
1	18	51.2	0	0	0	0	0	
2	20	69.2	0	0	0	1	0	Cysts of Glugea (*)
3	22.5	100.5	1	0	0	1	0	Cysts of Glugea (*)
4	24	111.9	0	0	0	0	0	
5	23.5	110.5	0	0	0	0	0	

Table 7. Overview of dab analysed five days after the treatment ("Test end" REF sub 1 to 3)

Sub-sample "Test-end REF1" 30 April 2014								CVI ID no. 14005712
Dab no.	Length (cm)	Weight (grms)	Ext haemor	Int haemor	Ext lesions	Int paras	Int lesions	Remarks
1	22.0	103.0	0	0	0	0	0	
2	20.0	60.7	0	0	0	0	0	
3	16.5	41.7	0	0	1	0	0	Big lesion ventral side, *Vibrio fortis–like* (*)
4	19.0	61.7	0	0	0	0	0	
5	22.0	99.7	0	0	0	0	0	
6	17.5	52.2	0	0	0	0	0	
7	22.0	99.7	0	0	0	1	0	Many cysts of *Glugea*
8	21.0	77.3	0	0	0	0	0	
9	17.0	48.2	0	0	0	0	0	
10	21.0	87.6	0	0	0	0	0	
11	20.5	73.1	0	0	0	0	0	
12	21.0	91.6	0	0	0	0	0	
13	17.5	54.1	0	0	0	0	0	
14	16.0	40.9	1	0	1	0	0	Small lesion ventral side near tail (*)
15	17.0	46.1	0	0	0	0	0	
16	21.0	80.1	0	0	0	0	0	
17	18.5	57.4	1	0	0	1	1	Red anus; haemor near mouth, many cysts of *Glugea* near anus

Sub-sample "Test-end REF2" 30 April 2014								CVI ID no. 14005712
Dab no.	Length (cm)	Weight (grms)	Ext haemor	Int haemor	Ext lesions	Int paras	Int lesions	Remarks
1	17.0	44.4	0	0	0	1	1	Many cysts of *Glugea*
2	18.5	63.5	0	0	0	0	0	
3	25.5	161.5	1	0	1	0	0	Lesion back side, haemor near tail (*)
4	21.0	84.3	0	0	0	0	0	
5	19.0	65.0	1	1	0	0	0	Hemor tail fin; lesions ventral side near tail fin, internal haemor near tail
6	20.5	82.4	0	0	0	0	0	
7	17.0	41.6	0	0	0	0	0	
8	21.5	85.8	0	0	0	1	0	Some cysts of *Glugea*
9	21.0	90.9	1	0	1	0	0	Eroded tail with haemor, 1 back lesion (*)
10	18.0	63.8	0	0	0	0	0	
11	16.0	37.1	0	0	0	0	0	
12	18.0	49.8	1	0	1	0	0	Eroded tail with haemor (*)
13	21.5	93.2	0	0	0	1	0	Many cysts of *Glugea*
14	26.5	187.9	0	0	1	0	0	Slighly eroded tail
15	20.5	77.1	0	0	0	0	0	
16	20.0	77.7	0	0	1	0	0	Slighly eroded tail
17	20.5	69.2	0	0	0	0	0	

Sub-sample "TEST-END REF3" 30 April 2014								CVI ID no. 14005712
Dab no.	Length (cm)	Weight (grms)	Ext haemor	Int haemor	Ext lesions	Int paras	Int lesions	Remarks
1	26.5	157.0	0	0	0	0	0	Swam belly up
2	27.0	166.0	0	1	0	1	1	Haemor aside gonads, swam belly up; cysts of *Glugea*
3	25.0	143.0	0	0	0	1	1	swam belly up; cysts of *Glugea*
4	17.0	45.0	0	0	1	0	0	Lesions at ventral fin, swam belly up (*)
5	21.5	99.4	0	0	0	0	0	No picture of ventral side
6	18.5	67.6	0	0	0	0	0	No picture of ventral side
7	23.5	112.0	0	0	0	0	0	No picture of ventral side
8	17.0	49.6	0	0	0	0	0	No picture of ventral side
9	22.0	88.6	1	0	0	0	0	Light haemor along side fin, no picture of ventral side
10	18.0	52.4	0	0	0	0	0	No picture of ventral side
11	18.0	52.1	0	0	0	0	0	No picture of ventral side
12	17.0	50.8	1	0	1	0	0	Fracture of side fin with haemor, no picture of ventral side (*)
13	24.5	119.0	0	0	0	0	0	
14	19.0	67.7	0	0	0	0	0	
15	18.5	59.6	0	0	0	0	0	
16	15.5	35.6	0	0	0	0	0	

Table 8. Overview of dab analysed five days after the treatment ("Test end" Delmeco sub 1 to 3)

Sub-sample "TEST-END DEL1" 30 April 2014								CVI ID no. 14005712
Dab no.	Length (cm)	Weight (grms)	Ext haemor	Int haemor	Ext lesions	Int paras	Int lesions	Remarks
1	23.0	112.2	0	0	0	0	0	
2	17.0	43.5	0	0	0	0	0	
3	18.5	59.3	0	0	0	0	0	
4	20.0	76.6	0	0	0	0	0	
5	21.5	85.9	0	0	0	0	0	
6	18.0	94.9	0	0	1	0	0	Eroded fin
7	23.0	106.3	0	0	0	0	0	
8	21.5	96.8	0	0	0	0	0	
9	17.5	48.0	1	0	0	0	0	Large subcutaneous haemor (*)
10	18.5	53.3	0	0	0	0	0	
11	21.0	85.5	0	0	0	0	0	
12	18.0	52.3	0	0	0	0	0	
13	18.0	51.6	0	0	0	0	0	
14	21.0	86.1	0	0	0	0	0	
15	17.0	45.3	0	0	0	0	0	
16	20.5	74.9	0	0	0	0	0	
17	18.0	61.7	0	0	0	0	0	

Sub-sample "TEST-END DEL2" 30 April 2014								CVI ID no. 14005712
Dab no.	Length (cm)	Weight (grms)	Ext haemor	Int haemor	Ext lesions	Int paras	Int lesions	Remarks
1	24.0	117.0	0	0	0	1	1	Many cysts of *Glugea*
2	22.0	94.5	0	0	0	0	0	
3	18.5	62.1	0	0	0	0	0	
4	22.0	95.4	0	0	0	0	0	
5	17.5	50.2	0	0	0	0	0	
6	17.5	48.6	0	0	0	0	0	
7	17.5	43.8	0	0	0	0	0	
8	18.0	59.8	0	0	0	0	0	
9	24.5	130.3	0	0	0	0	0	
10	16.5	42.8	0	0	0	0	0	
11	25.0	25.1	0	0	0	0	0	
12	21.5	92.2	0	0	0	1	1	Red anus, some cysts of *Glugea*
13	17.5	50.9	0	0	1	0	0	Starting lesions ventral side (*)
14	23.5	136.3	0	0	0	0	1	Muscle partly white
15	16.5	35.5	0	0	0	0	0	
16	18.5	62.1	1	0	0	0	0	Small petechial haemor ventral side
17	23.0	104.7	1	0	0	0	0	Small petechial haemor ventral side

Sub-sample "TEST-END DEL3" 30 April 2014								CVI ID no. 14005712
Dab no.	Length (cm)	Weight (grms)	Ext haemor	Int haemor	Ext lesions	Int paras	Int lesions	Remarks
1	24.0	119.1	0	0	1	0	0	Swam with belly up
2	18.5	59.1	0	0	1	1	1	Swam with belly up, many cysts of *Glugea*
3	20.5	72.1	1	0	0	0	0	Small petechial haemor ventral side
4	23.5	114.7	0	0	0	1	0	Cysts of *Glugea*
5	21.0	83.0	0	0	1	0	0	2x lesion ventral side (*)
6	20.5	80.7	0	0	1	0	0	2x lesion ventral side close to tail (*)
7	18.5	62.7	0	0	0	0	0	
8	21.5	81.9	0	0	0	1	0	Many cysts of *Glugea*
9	19.5	65.3	0	0	0	0	0	
10	20.0	82.1	0	0	0	1	0	Cysts of *Glugea*
11	24.5	133.7	1	0	0	0	0	Small haemor tail
12	18.0	53.1	0	0	1	1	0	Lesion back side, cysts of *Glugea* (*)
13	21.5	83.0	0	0	0	0	0	
14	21.0	77.4	0	0	0	0	0	
15	17.5	49.8	0	0	0	0	0	
16	16.5	38.5	0	0	0	0	0	
17	19.0	63.5	0	0	0	0	0	

Table 9. Overview of dab analysed five days after the treatment ("Test end" HFK sub 1 to 3)

Sub-sample "TEST-END HFK1" 30 April 2014								CVI ID no. 14005712
Dab no.	Length (cm)	Weight (grms)	Ext haemor	Int haemor	Ext lesions	Int paras	Int lesions	Remarks
1	19.0	64.2	0	0	0	0	0	
2	17.5	57.9	0	0	0	0	0	
3	19.0	58.0	0	0	0	0	0	
4	27.5	158.1	0	0	0	1	0	Many small cysts of *Glugea*
5	16.0	39.6	1	0	0	0	0	Haemor fins
6	17.0	42.5	1	0	0	0	0	Haemor tail fin
7	21.5	94.4	1	0	0	0	0	Haemor ventral side near the tail
8	23.0	117.1	1	0	0	0	0	Haemor (2) in fin bases
9	19.0	59.9	0	0	0	0	0	
10	19.5	64.6	0	0	0	0	0	
11	19.5	71.3	0	0	0	0	0	
12	17.5	49.5	0	0	0	0	0	
13	17.5	42.1	0	0	1	0	1	Pale fish, milky ascites, inflamed anus (*) *Vibrio anguillarum*
14	18.0	56.7	0	0	0	0	0	
15	23.5	113.3	0	0	0	0	0	
16	16.0	42.6	1	0	0	0	0	Petechial (spotlike) haemor fin and tail
17	17.5	50.7	0	0	0	0	0	

Sub-sample "TEST-END HFK2" 30 April 2014								CVI ID no. 14005712
Dab no.	Length (cm)	Weight (grms)	Ext haemor	Int haemor	Ext lesions	Int paras	Int lesions	Remarks
1	21.0	76.5	0	0	1	1	0	Lesion ventral side, many small cysts of *Glugea* (*)
2	17.0	49.8	0	0	0	0	0	
3	23.0	106.8	0	0	0	0	0	
4	19.0	68.4	0	0	0	0	0	
5	18.5	61.7	0	0	0	0	0	
6	21.5	92.1	0	0	1	0	0	Lesion ventral side (*)
7	17.0	46.5	0	0	0	0	0	
8	19.0	64.2	0	0	0	0	0	
9	24.5	127.8	0	0	0	0	0	
10	19.5	70.9	0	0	0	0	0	
11	18.0	60.5	0	0	0	0	0	
12	19.0	66.6	0	0	0	0	0	
13	22.5	94.8	0	0	0	0	0	
14	19.5	71.4	1	0	0	0	0	
15	16.5	41.2	1	0	0	1	0	Very small petechial haemor ventral side, some small cysts of *Glugea*
16	19.0	58.8	0	0	0	0	0	
17	17.5	55.8	1	0	1	0	0	Eroded tail; petechial haemor fin (*)

Sub-sample "TEST-END HFK3" 30 April 2014								CVI ID no. 14005712
Dab no.	Length (cm)	Weight (grms)	Ext haemor	Int haemor	Ext lesions	Int paras	Int lesions	Remarks
1	19.5	62.5	0	0	0	0	0	
2	19.5	57.6	0	0	0	0	0	
3	20.5	80.3	0	0	0	0	0	
4	20.0	62.5	0	0	1	0	0	Lesion back side (*)
5	20.0	69.7	0	0	0	0	0	
6	18.5	62.6	0	0	0	0	0	
7	22.5	98.8	0	0	1	0	0	Lesion back side (*)
8	20.0	65.4	0	0	0	0	0	
9	19.5	70.2	0	0	1	0	0	Lesion back side, "ring" mark at back side (*)
10	23.5	109.7	0	0	0	0	0	
11	24.0	124.6	0	0	0	0	0	
12	22.5	101.9	0	0	0	0	0	
13	21.5	86.6	0	0	0	0	0	
14	17.5	58.3	0	0	0	0	0	
15	16.0	34.1	0	0	0	0	0	
16	25.0	136.9	0	0	1	0	0	Delivered frozen; dead since 28 April, ext lesion ventral side scored at exposed

8 Appendix B Statistical analysis

8.1 Statistic methods

We hypothesed that fish injuries, measured immediately after treatment or 5 days later, were associated with the type of treatment (i.e. reference-REF, Delmeco-DEL and HFK) and some major biological features of the fish (i.e. length, weight and interval parasite status).

Test-post experiments

The "test-post" data are sorted per treatment category in Appendix A. Since the outcome of injury for each fish is in binary terms of presence (coded as 1) and absence (coded as 0), we assume that the outcome follows a binomial distribution. Therefore, theoretically a generalized linear model (GLM) with binomial distribution could be applied to model the probability of injury as a function of treatment type, fish length, weight and interval parasite status. The coefficients of the model are then estimated using a maximum likelihood estimation method (MLE). One important property of MLE for large samples is that its distribution tends to be normally distributed and approximates sample variances that can be calculated to generate confidence intervals. However, when the sample size is small, such as 5 fishes per treatment type in our experiments, MLE can be heavily biased and the condition of having a large sample does not apply. Another issue arises when we have a very small sample size is called the "complete separation" in the logistic regression, which is a situation where the explanatory variable "perfectly" predicts the response. For instance in our data, only 1 fish (exposed to HFK had internal parasite) exhibits "Ext haemor" injury, which apparently present a "perfect" correlation between HFK, internal parasite and "Ext haemor". However, this perfect correlation could be completely due to the small sample size. Due to these reasons, we decided to only report "the test-post" experiments results using descriptive statistics.

Test-end experiments

An example of the 5-day experiment data sorted per treatment category is given in Appendix A. The response Y_i ($i = 1, …, 151$) indicates the presence/absence of a specific injury after 5 days. However, since fishes were stored in nine tanks (around 17 fishes per tank), it is likely that the bacteria causing disease spread over a tank, or fishes get stressed from seeing injured congeners. Moreover, fishes from the same tank were exposed to the same biological environment, and such conditions are different among tanks, leading to varying injuring or healing conditions. As a result, the response of each individual fish Y_1, Y_2, …, Y_{151} is not independent of the response of another individual, as is required by a GLM. The experimental data exhibit a dependent/nested structure, indicated by the GroupID in the data. Variable GroupID indicates a subgroup of 17 fishes is kept together.

To account for the non-independent response of fish within one tank, we used a generalized linear mixed model (GLMM) model with GroupID as the random effect. We started with a full GLMM model (a model that includes all possible explanatory variables) as:

$Y_i \sim B(1, \pi_i)$
Full model: $\text{logit}(\boldsymbol{\pi}) = \boldsymbol{\alpha} + \boldsymbol{\beta_1} \times \boldsymbol{stimulus} + \boldsymbol{\beta_2} \times \boldsymbol{Int\_paras} + \boldsymbol{\beta_3} \times \boldsymbol{length} + a_i$ (1)

where Y_i indicates the binary response of a specific injury: $Y_i = 1$ if the injury was present and $Y_i = 0$ if the injury was absent in the observed fish i ($i = 1, …, 151$). Stimulus, "Int_paras", and length are the explanatory variables. Stimulus (REF, DEL and HFK), "Int_para" (yes and no) are categorical while length is continuous. A random effect ε_i ($i = 1, …, n$) contributes to the effect from each of the n subgroups (in this case n=9). The probability of injury given the values of the explanatory variables is defined as
$\pi_i = \Pr(Y_i = 1 | X_i = (stimulus_i, Int\_paras_i, length_i, a_i)$
Assumptions of this model are:

- Y_i follows a binomial distribution $B(1, \pi_i)$, with mean and variance properties $E(Y_i) = \pi_i$ and $var(Y_i) = \pi_i \times (1 - \pi_i)$.

- The logit of π_i, i.e. $\log\left(\frac{\pi_i}{1-\pi_i}\right)$, is a linear function of the explanatory variables.

- a_i follows a normal distribution with mean zero and variance $\sigma_a{}^2$

The optimal model coefficients $\boldsymbol{\beta_1}$, $\boldsymbol{\beta_2}$, $\boldsymbol{\beta_3}$ and α were then estimated using maximum likelihood estimation method (MLE). We then repeated such step for all sub models (see below). The optimal set of explanatory variables or the optimal model was then selected with the minimum Akaike Information Criterion (AIC).

Example of sub-models:
$$\text{logit}(\boldsymbol{\pi}) = \alpha + \boldsymbol{\beta_1} \times \boldsymbol{stimulus} + \boldsymbol{\beta_2} \times \boldsymbol{Int\_paras} \tag{2}$$
$$\text{logit}(\boldsymbol{\pi}) = \alpha + \boldsymbol{\beta_1} \times \boldsymbol{stimulus} + \boldsymbol{\beta_3} \times \boldsymbol{length} \tag{3}$$
$$\text{logit}(\boldsymbol{\pi}) = \alpha + \boldsymbol{\beta_2} \times \boldsymbol{Int\_paras} + \boldsymbol{\beta_3} \times \boldsymbol{length} \tag{4}$$
$$\text{logit}(\boldsymbol{\pi}) = \alpha + \boldsymbol{\beta_1} \times \boldsymbol{stimulus} \tag{5}$$
$$\text{logit}(\boldsymbol{\pi}) = \alpha + \boldsymbol{\beta_2} \times \boldsymbol{Int\_paras} \tag{6}$$
$$\text{logit}(\boldsymbol{\pi}) = \alpha + \boldsymbol{\beta_3} \times \boldsymbol{length} \tag{7}$$

Once the optimal model is chosen, 1) we present the p-value of each explanatory variable using the analysis of deviance (chi-squared) test. A significant p-value suggests an association between injury and the corresponding explanatory variable. The estimated coefficient indicates the magnitude of the effect. 2) When a non-significant p-value is obtained but the estimated coefficient is large, we then discuss the possibility of failing to detect a true effect (type II error).

Note that weight is not selected as an explanatory variable in the full model, due to its the high correlation with length (Pearson correlation coefficient ρ=0.92, Figure 13 below). Including highly correlated explanatory variables (collinearity) might lead to biased parameter estimates and over fitting of the model. Therefore, we excluded weight from the model.
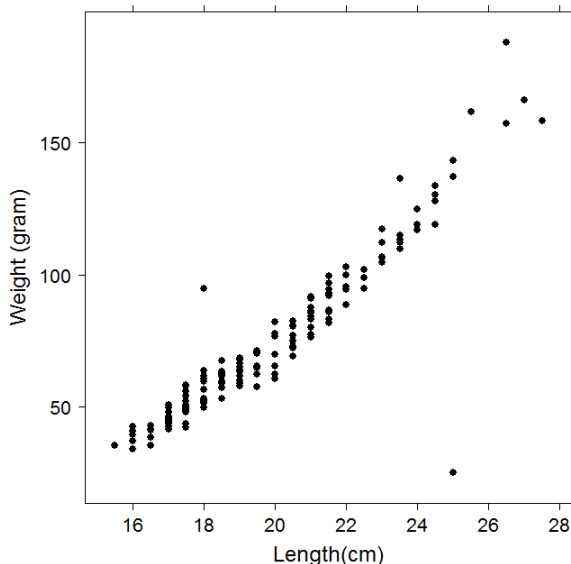

Figure 13. Scatterplot of weight vs. length.

9 Statistic results

9.1 Test-post group

Table 10. Frequency of injuries for each treatment type

Ext haemor	REF	DEL	HFK
No	5	5	4
Yes	0	0	1
Int haemor	REF	DEL	HFK
No	5	5	5
Yes	0	0	0
Ext lesion	REF	DEL	HFK
No	2	2	5
Yes	3	3	0
Int lesion	REF	DEL	HFK
No	4	3	3
Yes	1	2	2

Table 11. Frequency of injuries for "Int_paras" status

Ext haemor	Negative	Positive
No	11	3
Yes	0	1
Int haemor	Negative	Positive
No	11	4
Yes	0	0
"Ext lesion"s	Negative	Positive
No	7	2
Yes	4	2
"Int lesions"	Negative	Positive
No	10	0
Yes	1	4

9.2   Relation between length and injuries in the "test-post" group
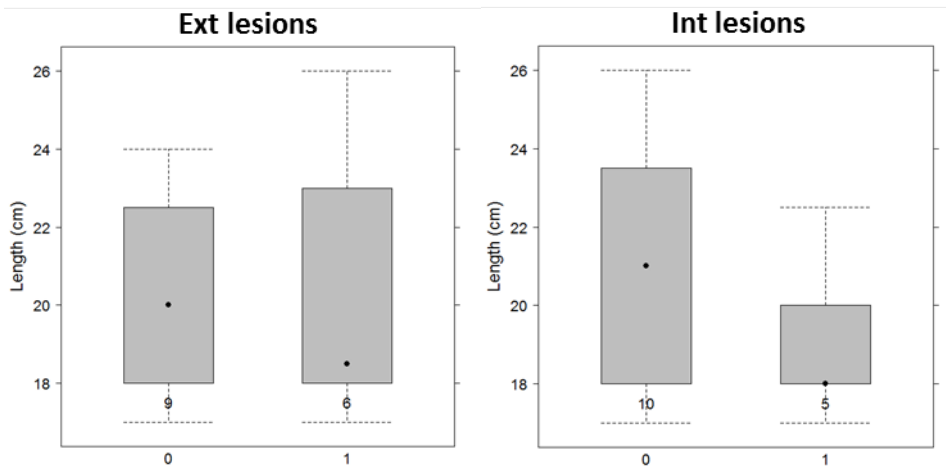
Figure 14. Boxplots of the length distribution for each injury type in the "test-post" group.

"Ext haemor" and "Int haemor" were hardly observed in the 15 fishes, irrespective of the stimulus type. Both REF and Delmeco treated fish had 3 out 5 with "Ext lesions", however, HFK treated fish did not end up with any "Ext lesions". Two out of 5 fishes from Delmeco and HFK had "Int lesion", while REF had only one fish with "Int lesion". All 4 fishes with positive internal parasites ended up with "Int lesions", which is suspicious and indicates a correlation. Fishes without "Int lesion" seem to have a larger length range or higher average length than fishes with "Int lesions". But this observation is not biologically sound, so the finding probably is due to chance. Despite all these descriptions, due to the small size sample, it is not possible to make any solid conclusions.

9.3   Test-end group

9.3.1   "Int paras" versus injury

Table 12. Frequency of injuries for "Int_paras" status

Ext haemor	Negative	Positive
No	115	15
Yes	19	2
Int haemor		
No	133	16
Yes	1	1
Ext lesion		
No	113	14
Yes	21	3
Int lesion		
No	132	10
Yes	2	7

9.3.2   Length versus injury

The relationships between fish length and the likelihood of injuries are illustrated in the 4 boxplots in Figure 15. The length distributions in the "test-end" groups show a different trend for "Int lesions" as compared to the "test-post" group, although not statistically significant. The observed shorter length with more internal lesion observed in the 15 fishes was probably due to chance.
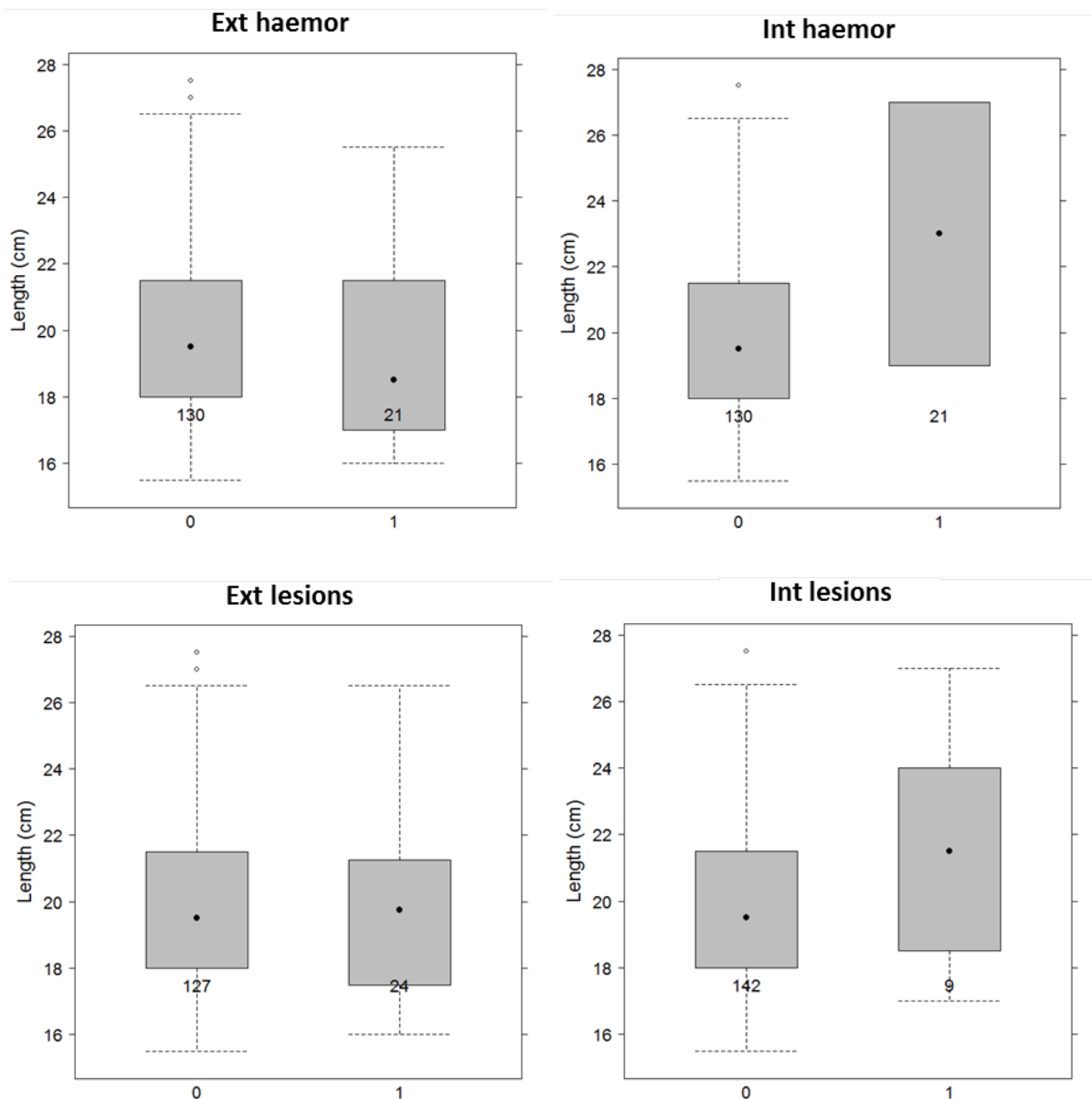
Figure 15. Boxplots of the length distribution for each injury type in the "test-end" group.

9.4 Response to pulse exposure as external haemorrhages

The AIC of model 7 was the minimum (AIC=126.8). However, model 6 (AIC=127.7) and model 5 (AIC=128.7) had only slightly larger AIC values. With such small AIC differences, it is difficult to statistically judge which model is optimal. One reason would be the weak association between injury and any of the explanatory variables, making it difficult to decide which one is the best one among all the bad models. Since we are interested in the stimulus effect in this study, we present the output of model 5. Analysis of deviance shows that stimulus factor obtained a non-significant p-value of 0.57 (Chi-squared test statistic: Chisq=1.14, degree of freedom: DF=2). Thus, we cannot reject that the null hypothesis that three stimulus types (including reference) have the same effect on injury. The estimated coefficients are also small, excluding the possibility of a type II error (Table 14).

Table 13. Frequency of "Ext haemor" injuries for each treatment type

	REF	DEL	HFK
No (injury)	42	46	42
Yes (injury)	8	5	8

Table 14. Statistical result of model 5

	coefficient	z-statistic	p-value	Chisq (DF)
Intercept	-1.66	-4.30	<0.01	
DEL vs. REF	-0.56	-0.92	0.36	Chisq=1.14
HFK vs. REF	$-5*10^{-5}$	0.00	1	DF=2

9.5   Response to treatment type as internal haemorrhage

Table 15 shows that the data show a complete separation. Therefore, we do not provide the output table with the estimated coefficients (because they are biased), instead we only provide the analysis of deviance. Analysis of deviance shows that the stimulus factor obtained a non-significant p-value of 0.11 (Chisq=4.48, DF=2).

Table 15. Frequency of "Int haemor" injuries for each treatment type

	REF	DEL	HFK
No	48	51	50
Yes	2	0	0

9.6   Response to treatment type as external lesion

Table 16. Frequency of "Ext lesion" injuries for each treatment type

	REF	DEL	HFK
No	41	44	42
Yes	9	7	8

Analysis of deviance shows that the stimulus factor obtained a non-significant p-value of 0.85 (Chisq=0.32, DF=2). The estimated coefficients are also small, excluding the possibility of type II error (Table 17).

Table 17. Statistical results of model 5

	coefficient	z-statistic	p-value	Chisq (DF)
Intercept	-1.53	-3.98	<0.01	
DEL vs. REF	-0.32	-0.57	0.57	Chisq=0.32,
HFK vs. REF	-0.14	-0.25	0.80	DF=2

9.7 Response to pulse exposure as internal lesion

Model 6 obtained the minimum AIC (AIC=49.5). On the other hand, the AIC for model 7 (AIC=71.6) and 5 (AIC=73.7) are much larger, suggesting that "Int paras" is a superior predictor for "Int lesion". With a p-value of <0.01 (Chisq 24.72, DF=1), the analysis of deviance shows that "Int paras" status was statistically significantly associated with "Int lesion", at the significance level of 0.05. The estimated coefficients are shown in Table 16. The large positive coefficient (4.09, or odds ratio of exponential(4.09) = 59.74) indicates that fishes with internal parasites are associated with a higher probability of "Int lesion" (Table 19).

Table 18. Frequency of "Int lesion" injuries for each "Int paras" status

	No_parasite	Yes_parasite
No (injury)	132	10
Yes (injury)	2	7

Table 19. Statistical results of model 6.

	coefficient	z-statistic	p-value	Chisq (DF)
Intercept	-4.42	-5.49	<0.01	
Int_paras Yes vs. NO	4.09	4.37	<0.01	Chisq=24.72, DF=1

Analysis of deviance shows that stimulus factor obtained a non-significant p-value of 0.29 (Chisq=2.49, DF=2). The estimated coefficients are also small, excluding the possibility of type II error (Table 21).

Table 20. Frequency of "Int lesion" injuries for each treatment type

	REF	DEL	HFK
No	46	47	49
Yes	4	4	1

Table 21 Statistical results of model 5.

	coefficient	z-statistic	p-value	Chisq (DF)
Intercept	-2.44	-4.69	<0.01	
DEL vs. REF	-0.02	-0.03	0.98	Chisq=2.49, DF=2
HFK vs. REF	-1.45	-1.28	0.20	

Justification

Rapport C171/14
Project Number: 4308601074

The scientific quality of this report has been peer reviewed by the a colleague scientist and the head of the department of IMARES.

Approved: B. van Marlen
                        Project Manager Fishing Technology
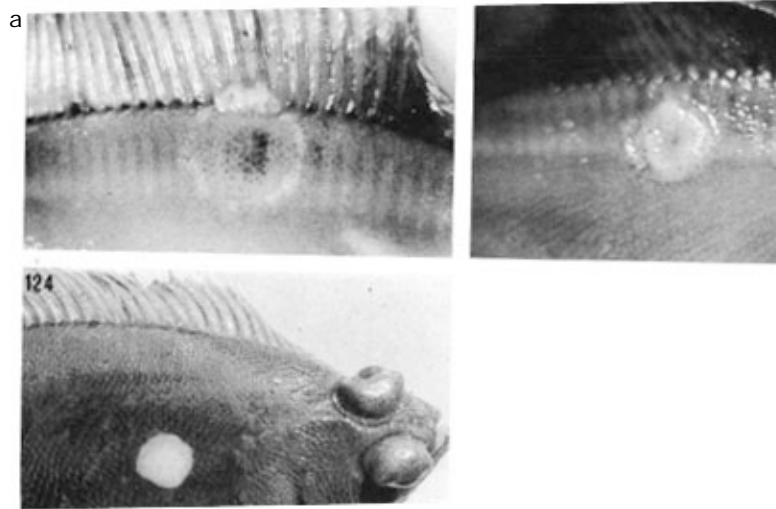
Signature:

Date: 22/05/2015

Approved: Drs. J.H.M. Schobben
                        Head of dept. Fish

Signature:

Date: 22/05/2015

10 Appendix C Illustrations

a

b)

Figure 1a and b. Lymphocystis virus (a) disease in dab (*Limanda limanda*) and papilloma in dab (b). (from Möller & Anders, 1983).

Figure 2. A sample of dab with ulcers and *Glugea*, taken from a pulse trawl catch and analysed by the CVI laboratory in May 2012 (ID no. CVI NL 12010586-9).
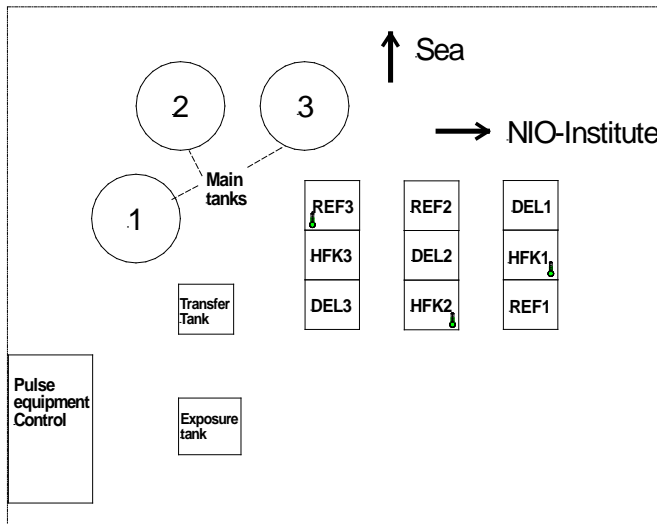
Figure 3. Overview of the fish holding system in the outer yard of the IMARES laboratory, Yerseke with the three main stores and the nine holding tanks with labels of exposed sub-groups.
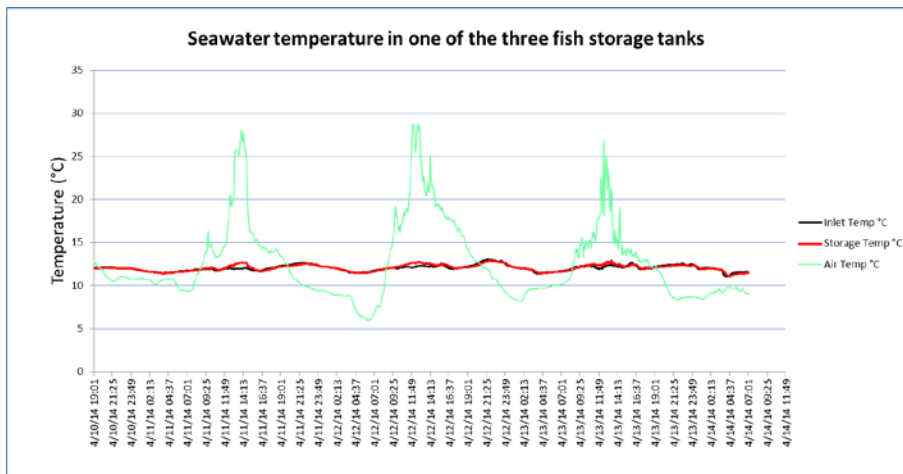
Figure 4. Temperature gradient of seawater in a main storage tank over three 24 hour cycles prior to the arrival of the dab at a water flow of 1750 l/h.
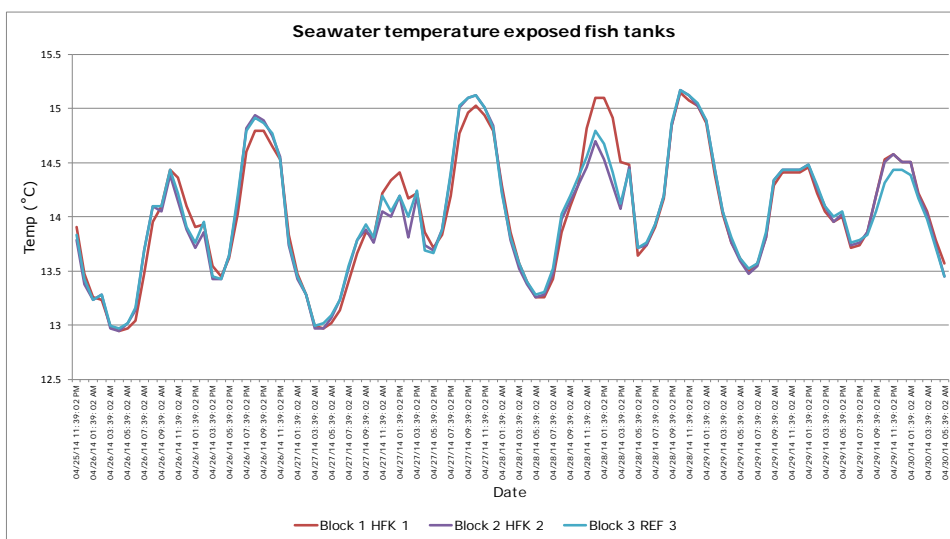
Figure 5. Seawater temperatures of three of the nine exposed fish tanks (Figure 3) over the post-exposure period.

Figure 6. Overview of the holding tanks and the exposure tank equipped with the HFK electrodes
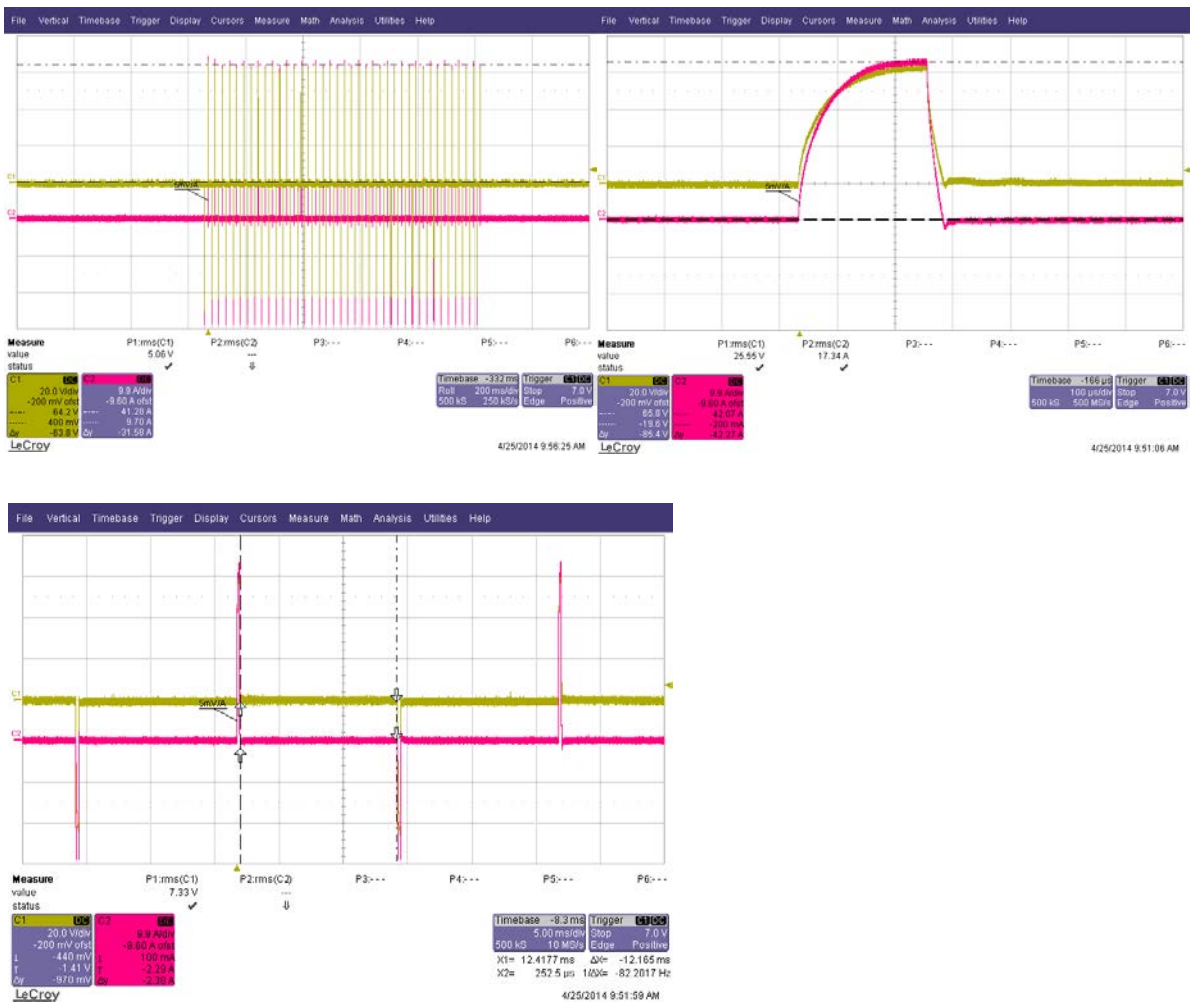
Figure 7. Delmeco pulse stimulus showing the burst of 39 bipolar pulses of 1 s total duration, the zoom-in on pulse voltage and current amplitude (62V/42A$^{0\text{-peak}}$) and pulse width of 250 µs, the bipolar pulse and the symmetrical division of pulse parts.
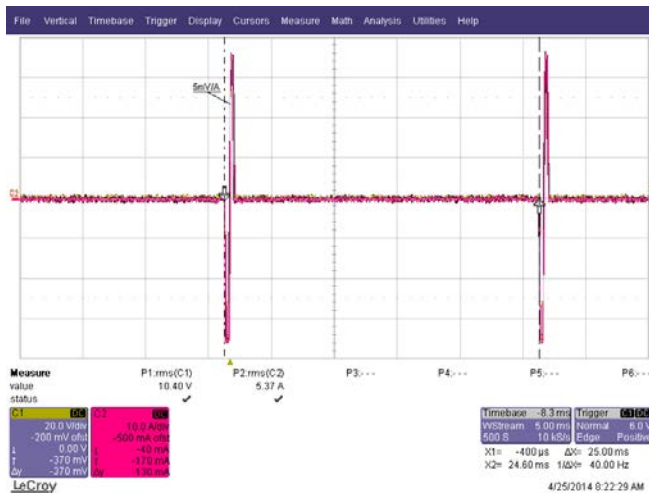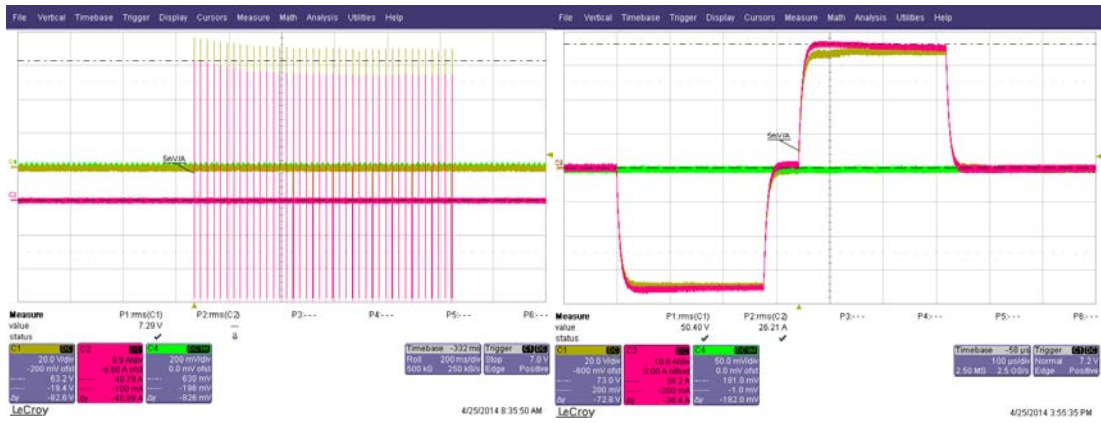
Figure 8. HFK pulse stimulus with a burst of 41 bipolar pulses of total duration 1 s, the zoom-in on a single pulse with voltage and current amplitude measured (76/70 V/36.4 A) and the measured full pulse cycle of 40 Hz.

Figure 9. Haemorrhagic lesion in dab before exposure to the HFK pulse stimulus. The fish was found dead the day after.

a)

b)

c)

Figure 10 a). *Glugea stephani* in dab of the "control" group, b) opened gut of *Glugea* infected dab with many cyst containing this parasite ,and c) a dab from the HFK treated group (HFK-1) with a red inflamed anus, due to a systemic *Vibrio anguillarum* infection. Internally, "milky" fluid was found between the internal organs.
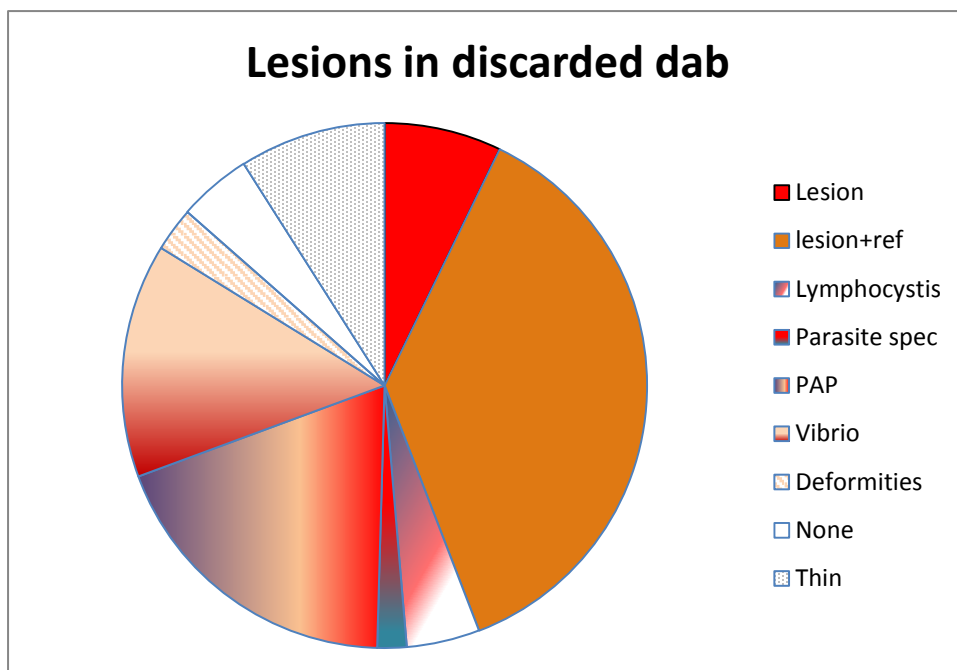
Figure 11. Contribution of lesions and diseases observed in discarded dab not taken to the laboratory. In the 57 discarded dab a number of 111 anomalies were found, and 5 dab had none. Category "lesion" reflects the fish with injuries without visible disease. The fish sorted as "lesion+ref" had a single or multiple diseases of which the share of types are shown separately. The diseases mainly refer to *Vibrio* spp, and epidermal hyperplasia/papilloma.
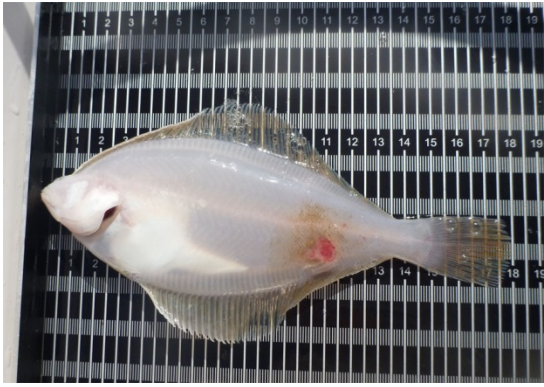
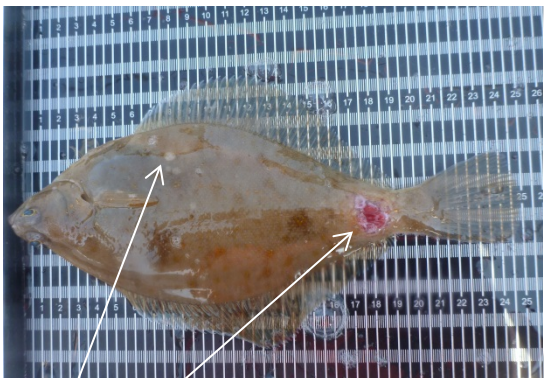a) Bacterial lesions, possibly by *Vibrio* spp. (B87)
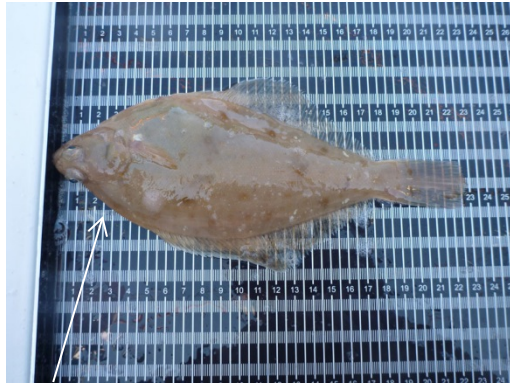
b) Epidermal hyperplasia/papilloma (B162)
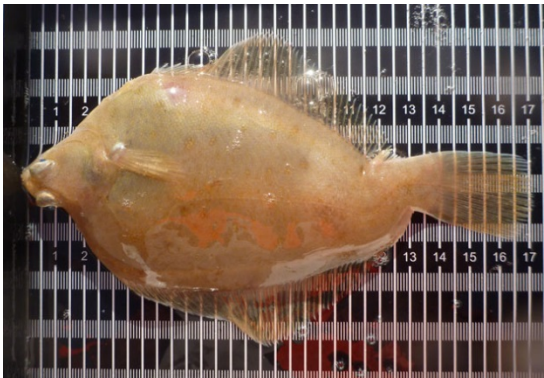
c) Deformed dab (B42)

d) Dab with Lesion (B32)

e) PAP & Vibrio (B250)

f) Dab with PAP & Vibrio (B204)

Figure 12a/e. Selection of 6 dab from the 57 discarded dab, with bacterial lesions, possibly *Vibrio* spp. (a, d, e, f), epidermal hyperplasia/papilloma (b, e, f), deformities (b, c, a), lesion (b), and lesions that show to be related to a combination of a bacterial lesion by e.g. *Vibrio* spp. and epidermal hyperplasia / papilloma (PAP) (e and f).