New species for EU aquaculture

Deliverable Report

Deliverable No:	D26.3	**Delivery Month:**	54
Deliverable Title	Determine immune response and effectiveness of orally delivered nodavirus capsid protein on protection of Atlantic halibut larvae		
WP No:	26	**WP Lead beneficiary:**	P7. IMR
WP Title:	Fish Health – Atlantic halibut		
Task No:	26.3	**Task Lead beneficiary:**	P7. IMR
Task Title:	Monitor and assess immune response and protection		
Other beneficiaries:			
Status:	Delivered	**Expected month:**	48
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Objective: The objective of this deliverable was to determine immune response and effectiveness of orally delivered nodavirus capsid protein on protection of Atlantic halibut (*Hippoglossus hippoglossus*) larvae.

Background: Atlantic halibut are affected by nodavirus especially at larval and early juvenile stages when the size of larvae is very small and the immune system is not well developed. Thus, classical vaccination regimes involving injection are not an optimal alternative. It is thus important to assess if it is possible to deliver the vaccine through feed during the late larval stages when it has been shown that the immune organs are well developed. If the immune system is functional and the delivery system can provide enough antigen concentration, this approach might offer protection against nodavirus, at least during the transition of live feed to commercial dry pellets at the end of metamorphosis.

Description:
The aim was to use the recombinant protein antigen expressed in different host systems either by injection intraperitoneally (*i.p.*) or by oral delivery. Proteins expressed in different host systems, in addition to different delivery systems, *i.e.* through live feed or direct application, might have an impact on the immune response for several reasons. Host cells might protect the recombinant antigen differently, first in the digestive system of the *Artemia*, then in the digestive system of the larvae, both of which might influence uptake of the antigen and where/how the antigen will stimulate the immune system.

Materials and methods:
The nodavirus capsid protein expressed in several different systems (***Deliverable 26.1 Assess the use of two eukaryotic expression systems; microalgae and a protozoan (Leishmania tarentolae) for production of nodavirus capsid protein***) was tested in ***Deliverable 26.2 Determine immune response and effectiveness of orally delivered VNN capsid protein on protection of Atlantic halibut larvae***. In the latter deliverable, if the *Artemia* tested positive for nodavirus capsid protein we checked if at least one of the oral delivery systems could work, as confirmed by a positive signal in larvae. To test whether the antigen delivered to the larvae can offer any protection, halibut larvae at 100 days post-hatch, just before they were weaned to commercial dry pellets, were chosen for use. *Artemia* were produced according to the standard protocol used at IMR and used for this purpose.

The treatment groups were as follows:

At the experiment start each treatment group had 50 larvae/juveniles.

1. Pichia extract expressing nodavirus capsid protein – oral delivery through *Artemia*
2. Pichia extract with empty vector, with no antigen - oral delivery through *Artemia*
3. Purified inclusion bodies of nodavirus capsid protein from *E. coli* with mineral oil adjuvant – *i.p.* injection
4. Purified VLPs from *Pichia* – *i.p.* injection
5. Purified VLPs from *Pichia* with adjuvant – *i.p.* injection
6. Purified nodavirus capsid protein expressed in tobacco leaves with mineral oil adjuvant – *i.p.* injection
7. Live *L. tarantolae* expressing nodavirus capsid protein - oral delivery through *Artemia*
8. Live *E. coli* expressing nodavirus capsid protein - oral delivery through *Artemia*
9. Purified inclusion bodies of nodavirus capsid protein from *E. coli* - oral delivery through *Artemia*
10. PBS with mineral oil adjuvant – *i.p.* injection
11. Negative control – non-treated

<u>For oral delivery (3 days in a row at end of June 2017):</u>

The larvae were starved before the first delivery of feed in the morning.

Early morning each day, the enriched *Artemia* were concentrated to 1000 *Artemia* per ml, washed and incubated at 20 °C for an hour so the *Artemia* open their jaws and are ready for feeding (**Figure 1**). At the end of incubation, the *Artemia* were mixed well and 35 ml per 50 ml tube was distributed. Either purified protein or live organisms expressing recombinant capsid protein were added to the respective treatment tubes, and the tubes were incubated in a water bath with aeration to maintain 20 °C during the feeding period with *Artemia*.

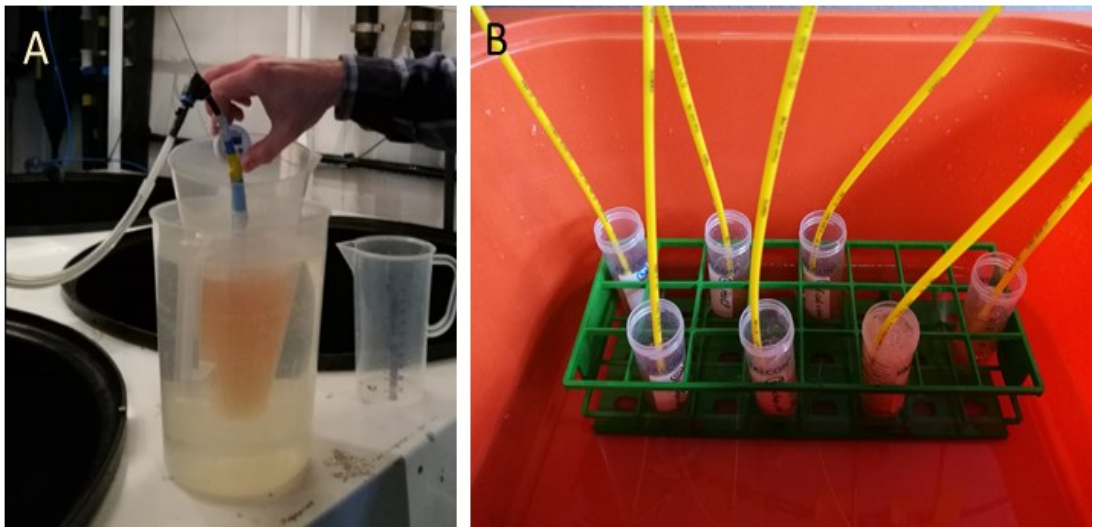

Figure 1. (A) *Artemia* incubated in water bath before being used for feeding specific recombinant capsid protein, (B) Tubes with *Artemia* with aeration during incubation for uptake of specific protein or live organisms for oral delivery.

At the end of the incubation period, the *Artemia* were filtered through a plankton mesh, washed once with sea water and the specific *Artemia* were added to the respective treatment tanks containing as little as 15 L seawater per tank. The larvae/juvenile halibut were allowed to feed on the *Artemia* carrying nodavirus capsid protein for 4 h with aeration in the tanks (**Figure 2**).

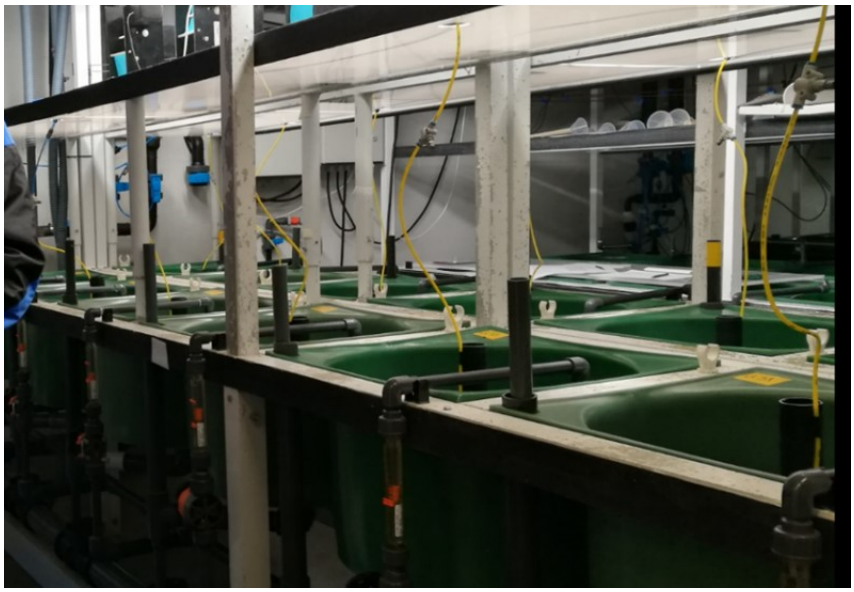

Figure 2. *Artemia* that had been fed with nodavirus capsid protein expressed in different systems were fed to halibut larvae/juveniles and the tanks were aerated during the feeding period.

At the end of feeding period, the flow through of water in the tanks with no GMO was started. In the tanks with GMO, flow through was also started but the water from these tanks was collected in a specially built extra storage tank and treated with chlorine following the authorized GMO application connected to this study.

The larvae/juveniles were given one feed portion of routine enriched *Artemia* late in the evening and starved in the morning to repeat the feeding with *Artemia* given the different antigens in the afternoon. The process was repeated for 3 days in a row. On the second day after the experiment start, the treatment groups to be injected intraperitoneally (*i.p.*) received single injections (**Figure 3**) after sedation, and then were transferred back to their respective tanks. The treatment that included adjuvant for *i.p.* injection was visible in the peritoneum of the larvae.

Figure 3 Halibut larvae/juveniles *i.p.* injected with VNN capsid protein expressed by different systems and formulated with mineral oil adjuvant. The peritoneum confirms the correct delivery in the form of white traces in the peritoneum. The top two photos depict larvae during sedation in small trays, while the bottom two photos show larvae/juveniles from two different injection treatments after they were moved back to their respective tanks. Larvae/juveniles varying in their size and developmental phase can be seen within and between the treatments.

The larvae/juveniles used for vaccination were in varying developmental phases, and this combined with the treatment and handling, resulted in several larvae/juveniles dying during the first few days. At the end of 10 weeks of vaccination, the juveniles that had survived within each treatment were transferred to the wetlab challenge facility, IMR, Bergen and acclimatized for 10 days. A few juveniles died due to the transport and handling process. The number of individuals that survived within each treatment group at the time of challenge thus varied from 9 – 20. In the non- treated group, there were 29 individuals that survived.

Half of the individuals in the non-treated groups were sedated and challenged with nodavirus by *i.p.* injection of 50 µl at $1x10^{7.5}$ $TCID_{50}$ /ml and transferred to a new tank resulting in one non-treated non-challenged group and one non-treated challenged group to enable comparison with the rest of the vaccinated challenged treatments. In the other treatment groups, all individuals were i.p. challenged with a similar dose of nodavirus per individual. The experiment was terminated in week 50 2017, and all fish were sampled for brain and spleen. Brain samples were analysed for nodavirus using a RNA2 specific real time rt-PCR assay (Korsnes et al, 2005) to assess the effect of different vaccination treatment.

Results:

The individuals within each treatment group and between treatment groups were in varying developmental phases and accordingly were expected to have different weights at the start of the experiment. During the experiment weight and development of the individuals continued to differ and this was reflected in the large individual differences seen at the end of the experimental period (**Figure 4**). Several individuals weighing below 7-10 g still had the appearance of late larval stages, while individuals above 15-20 g showed signs of successful metamorphosis and migration of eyes.

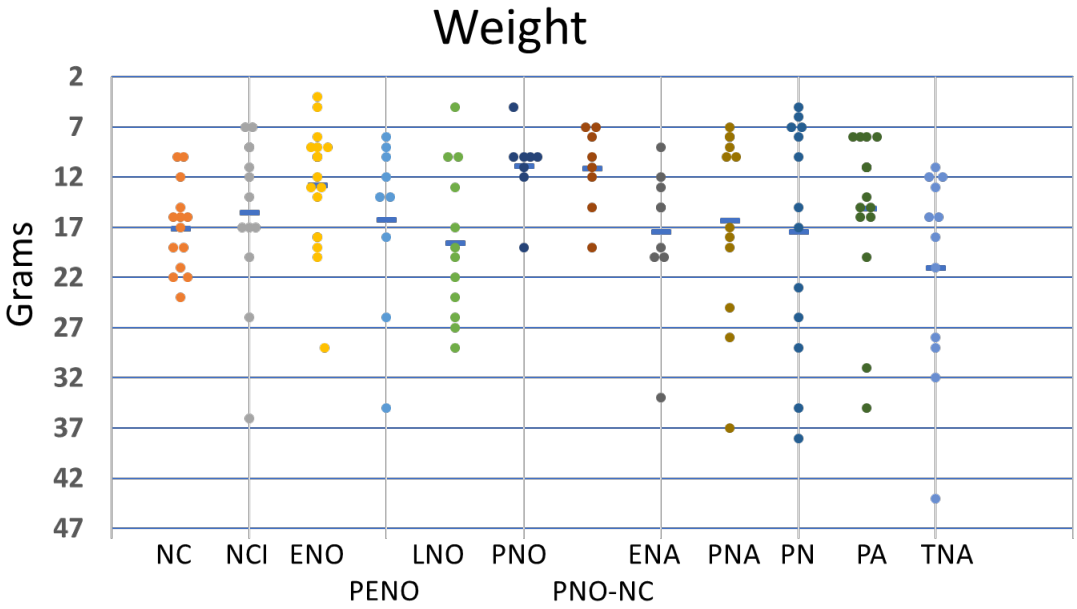

Figure 4 Weight of larvae/juveniles at the last sampling time-point. Each individual has been shown along with the median value (blue horizontal dash). NC – Non vaccinated negative control, NCI – Non vaccinated control challenged, ENO – Live *E. coli* expressing capsid protein orally delivered through *Artemia*, PENO – Purified inclusion bodies containing nodavirus capsid protein expressed by *E. coli* delivered through *Artemia*, LNO - Live *L. tarentolae* expressing capsid protein orally delivered through *Artemia*, PNO – Freeze fried Pichia without nodavirus capsid protein orally delivered through *Artemia* as Pichia negative control, PNO-NC – Freeze dried Pichia expressing capsid protein orally delivered through *Artemia*, ENA - Purified inclusion bodies containing nodavirus capsid protein expressed by *E. coli* formulated with mineral oil and delivered by *i.p.* injection, PNA – VLPs expressed by Pichia formulated with mineral oil and delivered by *i.p.* injection, PN - VLPs expressed by Pichia without mineral oil and delivered by *i.p.* injection, PA – PBS with adjuvant as negative control for *i.p.* delivery, TNA - Purified nodavirus capsid protein expressed in Tobacco leaves formulated with mineral oil and delivered by *i.p.* injection.

The Ct values of the RNA2 of nodavirus in the different groups is shown in **Figure 5**. The treatment groups showed no difference in protection. The treatment groups that were non-vaccinated and not treated, together with the one that was injected with PBS formulated with adjuvant, showed almost similar amounts of viral RNA2 compared to the vaccinated groups. When comparing vaccine delivery systems oral to injection and with adjuvanted injected groups, the adjuvanted injected groups showed slightly lower amounts of virus (1-3 Ct values), and a single individual in most of the injected groups had very little virus.

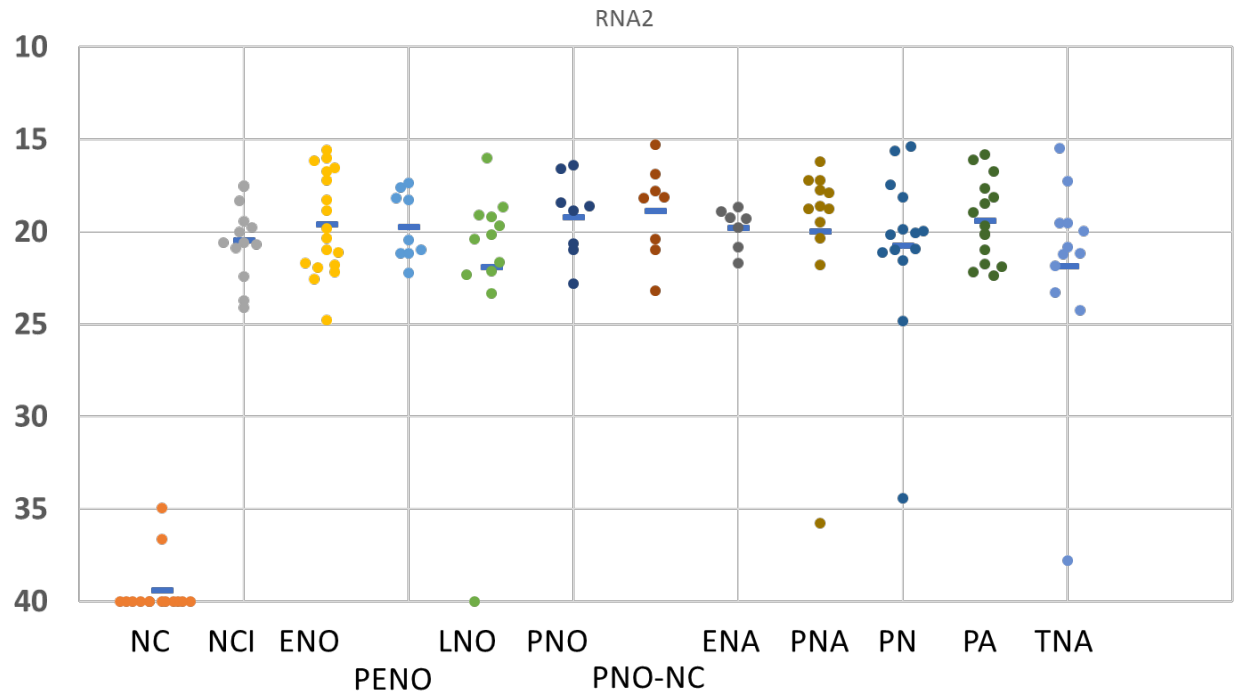

Figure 5. Ct values of RNA2 analysis of all the experimental groups. Each individual has been shown along with the median value (blue horizontal dash). NC – Non vaccinated negative control, NCI – Non vaccinated control challenged, ENO – Live *E. coli* expressing capsid protein orally delivered through *Artemia*, PENO – Purified inclusion bodies containing nodavirus capsid protein expressed by *E. coli* delivered through *Artemia*, LNO - Live *L. tarentolae* expressing capsid protein orally delivered through *Artemia*, PNO – Freeze fried *Pichia* without nodavirus capsid protein orally delivered through *Artemia* as Pichia negative control, PNO-NC – Freeze dried *Pichia* expressing capsid protein orally delivered through *Artemia*, ENA - Purified inclusion bodies containing nodavirus capsid protein expressed by *E. coli* formulated with mineral oil and delivered by *i.p.* injection, PNA – VLPs expressed by Pichia formulated with mineral oil and delivered by *i.p.* injection, PN - VLPs expressed by Pichia without mineral oil and delivered by *i.p.* injection, PA – PBS with adjuvant as negative control for *i.p.* delivery, TNA - Purified nodavirus capsid protein expressed in Tobacco leaves formulated with mineral oil and delivered by *i.p.* injection.

Discussion

The challenge experiment was terminated at 8 weeks post infection, and no mortality was observed during this period. It is normally very difficult to achieve mortality in a challenge model for nodavirus for Atlantic halibut larvae of this size or larger, which is different from many other fish species. Thus,

the amount of virus in brain tissues has been used as an indicator of the protection achieved by vaccination (Øvergård et al, 2013). We aimed to test possible protection to nodavirus infection by oral delivery of the nodaviral antigens as this has been shown to induce an immune response in different fish species, and in some cases a higher survival rate was observed (Reviewed in Yong et al 2017). Several different ways for oral and bath delivery have been tried including through the use of live feed *Artemia* for larval stages.

The RT-qPCR analysis of brain samples shows that the control fish (not challenged, NC) were negative (Ct value of 40), indicating that the population of larvae used in this experiment had been free of nodavirus when the experiment started. A relatively high amount of nodavirus RNA2 was detected in the juveniles that were not- treated and later challenged (NCI) confirming that the challenge model worked. The amount of virus detected in the treatment groups was quite similar to the non-vaccinated challenged control (NCI), apart from a few individuals where the Ct <30. The size of the individuals in vaccinated and non-vaccinated groups at the start of the experiment was comparable but with large individual variation, and several larvae did not show signs of the end phase of metamorphosis (**Figure 3**).

Earlier studies have shown that at 94 days post hatch just before the transition from live feed to commercial dry pellets, the larvae have a developed immune system where also IgM^+ (B) cells could be detected using immunohistochemical analysis (Patel et al, 2009; Øvergård et al, 2011). Thus, most larvae were expected to have a fully functional immune system at the time of vaccination. However, apart from the observation that the adjuvanted injected groups showed a slightly lower amount of virus, none of the treatments gave any protection to the juveniles. In general, it seems that the larvae did not respond to the vaccination at all, nor did the individuals develop tolerance (as higher viral amounts in vaccinated groups were not observed). There might be several reasons for these findings. As mentioned earlier, most larvae were underdeveloped for the phase they were in, and very few had reached the expected developmental stage. The oral treatments were carried out once a day for three days in a row, and based on the results in Deliverable 26.2, we could see that the capsid protein was broken down into smaller proteins in *Artemia*, and thus we do not know if the larvae received enough antigen and if the antigen was in the right conformation to induce a protective immune response. If delivering the antigen for a longer period would have had a better effect is unknown. The antigenic formulation with and without adjuvant that was delivered by *i.p.* injection did not go through the same route via *Artemia* and hence could have given protection. However, it can be speculated that due to the very small peritoneum it was extremely difficult to deliver enough antigen, and most larvae received around 10-20 µl of either pure antigen or adjuvanted antigen, and thus the concentration of antigen was much lower than planned. In an earlier study using one of the formulations, adjuvanted purified capsid protein expressed in *E. coli*, when delivered by *i.p.* injection to halibut weighing approximately 25 g elicited protection in most of the juveniles (Øvergård et al., 2013). The same formulation that was used as a positive control in this study did not give any protection to larvae, leading us to speculate that the amount of antigen that was delivered could have been too low.

Testing these antigens in larvae that are sorted such that all individuals are in the same developmental phase or by delivering the antigens along with dry pellets rather than through *Artemia* would reveal if the antigens can give protection at a stage earlier than at 25 g.

Since none of the vaccination treatments showed any protection, the spleen was not analysed to assess the immune genes involved in adaptive immunity. This analysis would not have given any further understanding to the results obtained and would simply have reconfirmed that all groups had little or

no specific immune response. Also, the brain samples that were collected for histology and IHC were not analysed for the same reason.

Conclusion:
Although it has been shown that *Artemia* will take up and accumulate the various forms of recombinant nodavirus capsid proteins and act as a vector for oral delivery to larvae of Atlantic halibuts, it can be concluded from the challenge experiments that this strategy of antigen delivery does not induce protection against nodavirus infection, at least under the conditions used in this study.

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Deviations: None

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