Vitenskapskomiteen for mat og miljø
Norwegian Scientific Committee for Food and Environment

Response to the Norwegian Environment Agency from VKM Your ref.: 2023/5243

Additional considerations on the environmental risk of genetically modified sterile VIRGIN® Atlantic salmon for use in research trials in aquaculture sea-cages

Background

In its report 2023:20, the VKM delivered its risk assessment of a research trial with genetically modified sterile VIRGIN® Atlantic salmon (*Salmo salar*) to be released in aquaculture sea-cages. The research material was F1 juveniles whose F0 parents were micro-injected with CRISPR/Cas9 to knock out the *dnd* gene necessary for the formation of germ cells, and co-injected with *dnd* mRNA to make the F0 parents fertile. Mosaïcism in the 2-male-by-4-female F0 parents that were crossed created 303 *dnd*-knockout (KO)/*dnd*-knockout individuals which together with 485 wildtype/wildtype individuals were intended for release at the smolt stage into four 5 x 5 m nets within a larger net at the IMR Matre Research Station, Western Norway.

VKM assessed that the research trial was associated with a potentially high risk to wild Atlantic salmon populations. VKM assessed that the information (single run genotyping data and methods related to this) received from the applicant could not exclude the possibility that potentially heterozygous fertile salmon existed among the 303 putative sterile *dnd*-KO/*dnd*-KO and that potentially heterozygous carriers of a sterility allele existed among the fertile 485 putative wt/wt salmon.

Additionally, VKM assessed that the use of double netting in sea cages with smaller nets anchored within a larger net did not necessarily reduce the likelihood of experimental fish escaping from the research facility. Hence, VKM concluded that an unknown number of the experimental fish could potentially introduce sterility alleles into wild salmon populations if such individuals escaped from the experiment.

VKM suggested five measures to reduce risk or uncertainty of the research trial (chapters 5 and 6 in VKM 2023:20):

I. VKM noted that many different knockout mutations were found among the 303 *dnd*-KO/*dnd*-KO fish and that very few individuals of each knockout mutation had been analysed with respect to confirmation of sterility.

II. Confirmative genotyping of fin-clips from the chosen 303 *dnd*-KO/*dnd*-KO and 485 wt/wt fish. This was considered necessary to reduce the potential of errors from handling and characterizing the 2200 samples that were used to select them.
III. An external marker on all PIT-tagged individuals to ease the recapture (and knowledge) of experimental fish; should they escape.
IV. A broader vaccine approach and a strategy to harvest experimental data by measuring the response in test and control groups.
V. Experiments in closed facilities that could have informed the risk assessment with respect to ecological (competitive and predator-prey) interactions with wild fish.

Based on the risk assessment by VKM, the applicant was requested by the Environment Agency to specifically provide data confirming the genotypes (re-genotyping) of all experimental fish and data confirming that the identified various genotypes of *dnd*-KO/*dnd*-KO fish results in phenotypically sterile (germ cell free, GCF) fish. I.e., that a relevant number of fish representing each identified KO-genotype be sacrificed in order to provide morphological and histological analyses confirming sterility. In addition, the applicant was asked to provide gene-expression levels of the *dnd*-gene and the gene-marker *vasa,* for each genotype.

Additional data addressing the other risk-reducing measures identified by VKM (III, IV & V listed above) were not specifically requested by the Norwegian Environment Agency. However, the applicant was asked by the Norwegian Environment Agency to provide any data that would help reduce uncertainties.

Mandate from the Norwegian Environment Agency for additional risk assessment

The Environment Agency has in an assignment of April 8th, 2024 asked VKM to assess whether the new information from the applicant (dated February 29th, 2024 and March 8th, 2024) changes the conclusions VKM presented in VKM Report 2023:20.

New data supplied from the applicant

Upon request by the Environment Agency the applicant has supplied four datasets based on additional studies:

1. Re-genotyping of all individuals that could be included in the experiment (n=277).
2. Gene expression of *dnd* and *vasa* (n=61).

3. Histology of gonad tissue of individuals that had their genotype confirmed in the second round of genotyping (n=61).
4. Gross morphology from the same 61 individuals.

In addition, the applicant has opted to replace the 485 wt/wt controls with conventional cultivated salmon of similar age, as a risk-reducing measure.

During assessment of the new data, VKM had additional questions regarding the supplied datasets and interpretation. Thus, a Teams meeting was held on April 15th involving the applicant (including relevant experts), VKM and Even Thoen (Patogen), to clarify uncertainties related to the supplied datasets. One of the topics discussed in this meeting was the discrepancy between 303 and 277 fish intended as trial candidates, and the applicant offered two veterinary reports to explain this reduction.

VKMs assessment of the new data

1. Regarding re-genotyping

On October 17th, 2023, a 277 of the 303 putative *dnd*-KO/*dnd*-KO fish remained, of which 276 were re-genotyped by the applicant (one fin-clip lost, and the fish was killed).

Twenty-seven of these (10 %) showed a different genotype than found in the first MiSeq analysis. Among these, 24 had the new genotype "ins22", which was not discovered in the first analysis, and three had wt alleles. Of the three potentially fertile (carrying wt alleles) individuals, one was a heterozygote carrying a knockout allele and a wildtype allele and showed one developing gonad when opened. Another was a heterozygote with one knockout allele and one mutated allele without a frameshift. The third was a wt/wt homozygote. Hence, one or potentially two of the re-analysed fish were individuals with a genotype which the VKM Report 2023:20 concluded must not exist among any research trial individuals released in locations where escape events may occur. See Figure 1 for an overview of how these were identified.

Sub-conclusion: From these results VKM concludes that there was a significant error rate in the initial genotyping, but that this second round of genotyping reduces the uncertainty regarding the genotype of the remaining 185 possible trial candidates.

2. Regarding gene-expression data

qPCR of *dnd* and *vasa* from the 61 knockout individuals showed very limited (or no) expression, whereas these genes are expressed in wildtype controls. The limited expression found was explained as "background" levels in knockout individuals (this may also occur in tissues where these genes are not expected to be expressed). At the Teams meeting on April

15th VKM requested information on "limit of detection" for the target genes analysed by the qPCR (i.e., quantification cycle (Cq-value) cut-off) and received a written reply with attached protocols by Wednesday April 17th. VKM was satisfied with this additional information.

Sub-conclusion: The qPCR data strengthens the applicant's claim that the *dnd*-KO genotypes included in the analyses do not have expression of *dnd*, and thus indicate sterility.

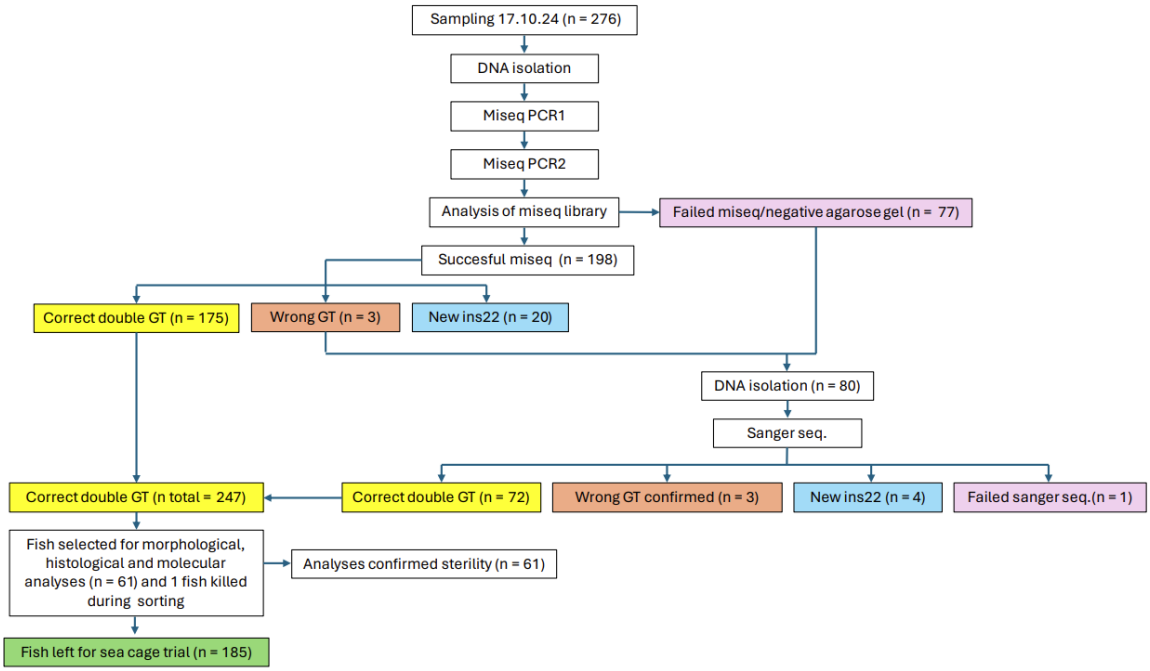

Figure 1: Schematic overview of workflow and result for samples included in the double genotyping, as provided by the applicant. The date of sampling 17.10.24 must be an error for 17.10.23.

3. Regarding histology data

The new histological data for the 61 individuals, in addition to previous histological data provided earlier, covers all the common *dnd* KO mutations (del 8, del 7, del 10, del 5 and ins1). The distribution of the different genotypes of the 61 individuals, i.e., the number of fish with each genotype: del10/del10 = 4; del7/del10 = 4; del10/del5 = 5; del7/del5 = 5; del8/del10 = 12; del8/del5 = 12 and del8/del8 = 19, respectively.

VKM requested external assistance from histopathologist Even Thoen, Patogen AS (see attachment) to interpret the histological data that was submitted to the Environment Agency as a data file with histological preparations and a link to a program for studying the information. The conclusion from Patogen AS was that all the checked individuals (except

wildtype controls) were assessed as sterile based on the absence of normal tissue structures of the gonads and no observation of oocytes/oogonia (females) or spermatogonia (males). Some residual uncertainty regarding knockout males was noted because epithelial cells in the gonads could not be easily distinguished morphologically from spermatogonia. This residual uncertainty was addressed by associate professor Rüdiger Schultz, Utrecht University (and researcher II at IMR, Bergen) in a Teams meeting with the applicant on April 15th:

> "*Spermatogeneic tubules from germ cell-free animals show only Sertoli cells. With increasing age, some of the males show continuing proliferation activity of the Sertoli cells, such that several layers of Sertoli cells accumulate. Since blood vessels in testis tissue are never present in the tubules but only found in the interstitial tissue, Sertoli cell groups ending up in the center of several cell layers get increasingly malnourished and hypoxic, such that necrotic cell loss becomes visible".*

His conclusion that this represents proof of sterility in knockout males was accepted by Even Thoen at the meeting, and consequently by VKM as evidence of sterility in the 61 individuals.

However, in the table listing all remaining candidates to be used in the trial (n=185), VKM found that 22 individuals had *dnd* mutations (del2, del4, del28, ins5 or ins10) alone or in combinations with other knockout alleles that was not represented among the 61 individuals that have been analysed by qPCR, gross morphology, and histology. No confirmation regarding the sterility of these 22 individuals was thus presented by the applicant.

Sub-conclusion: In a Teams meeting with the applicant on April 15th the applicant agreed with VKM that the 22 individuals without confirmational data should be removed from the list of knockout individuals to be included in the trial. VKM concludes that there is now low uncertainty regarding the sterility of the fish carrying genotypes included in the histological analysis.

4. Regarding gross morphology

The applicant has supplied individual photos of each of the 61 fishes used to collect additional data. The photos show the inside of the fish after removal of most internal organs.

Sub-conclusion: Histology has already confirmed that the individuals included here are sterile. VKM thus finds that the supplied photos of gross morphology do not add any additional information.

Aspects covered in the original risk assessment (VKM Report 2023:20) that have not been addressed by the applicant with new data

Although not specifically requested by the Norwegian Environmental Agency, the applicant was asked by the Norwegian Environment Agency to supply any relevant data that would help reduce uncertainties, e.g., regarding risk-reducing measures identified in the VKM report 2023:20.

VKM has not received information on whether or not PIT-tagged experimental fish will be marked externally to ease the recapture (and knowledge) of these, should they escape. Nor is VKM aware of a plan beyond traditional measures used in aquaculture for recapture of experimental fish, even though the experimental facility is fenced and marked as a GMO (Genetically Modified Organisms) experiment.

VKM has not received data describing a broader vaccine approach or a strategy to harvest experimental data by measuring the response in test and control groups. This lack of information does not influence the risk assessment but could lead to more information if the experimental material were challenged by parasites or other disease agents. The information given about the moderate mortality in summer 2023 after transfer to a sea water tank is sparse (see separate considerations regarding this mortality).

VKM has not received data that could have informed the risk assessment with respect to competitive and predator-prey interactions with wild fish. Experiments analysing ecological threats to wild salmonids (e.g., behaviour related to migration, aggression or other behaviour on spawning sites, and predatory behaviour towards smaller wild fish) would have reduced the uncertainties for the risk characterization, but not necessarily risk to wild populations. Protocols exist on how to obtain such information in closed facilities.

Additional assessments and considerations by VKM

VKM wants to point out the severity of the quite large discrepancy between the original and the re-confirming genotyping analyses. The second genotyping demonstrates not only the importance of a confirmative genotyping, but also the need for a thorough validation of all possible allelic variants that must be detected by the genotyping protocol. In this case, the ins22 allele was not detected in the original genotyping, and as a result, individuals carrying this allele were defined as homozygotes (del10/del10, del7/del7, del8/del8 and del2/del2) in the original MiSeq. The applicant explained this discrepancy by:

> "*The primer pair used in the first genotyping efficiently detected the wt allele and common indels while longer inserts in the dead-end gene were likely not as efficiently amplified by these primers*."

Hence, 24 of the 276 *dnd*-KO/*dnd*-KO individuals must now be reclassified from being homozygotes for a given mutated *dnd* allele to having two different mutated *dnd* alleles, one of which being ins22. Based on the frequency of this genotyping error found for 276 *dnd*-KO/*dnd*-KO individuals, VMK finds that it would not have been unlikely to find individuals carrying an ins22 allele amongst the 485 wt/wt individuals that were originally intended as controls. No explanation has been provided for the three individuals that changed category.

The applicant originally claimed that the cohort contained 303 *dnd*-KO/*dnd*-KO and 485 wt/wt individuals, which now has been disproven. Most likely, the cohort applied for originally contained several individuals that were heterozygote carriers of a *dnd*-KO allele, with a potential to spread this *dnd*-KO allele to the wild salmon population in the event of an escape. Now, the 485 wt/wt were taken out of the experiment before confirmative genotyping and replaced with conventional farmed salmon. The 24 individuals with ins22 and 3 individuals that changed category, have also been taken out.

Taken together, this leaves VKM with the impression that the combination of CRISPR-generated F0 generation giving rise to an F1 generation with a multitude of *dnd*-knockout (and other) alleles, and a sequencing technology that gives variable results as to the status of F1 individuals, is not trustworthy with respect to generating correctly classified research material.

Regarding the discrepancy from 303 to 277 experimental fish

VKM noted that the number of trial candidate fish was reduced from the original 303 individuals to 277 individuals. This was discussed with the applicant in the meeting on April 15th. In the meeting the applicant stated that the mortality was a result of de-smoltification (and consequently, lack of seawater adaptation) after the normal period for smolting and that mortality occurred both in experimental fish and control fish, with no apparent variation between type or body size. However, a veterinary report from "Sjukebesøk" July 7th, 2023 stated:

> "*På besøksdagen blei det obdusert og prøvetatt 4 fisk (2 døde og 2 svimarar = alt som var av dødfisk og svimarar). Det var sår på ein svimar og ein dødfisk og elles småblødningar på indre organ (tegn på sepsis). Det blei utført bakteriologisk dyrking på blodskål med salt frå nyret til dei 4 fiskane. Frå dei to fiskane med sår blei det vekst av hemolyttisk blandingsflora. Bakteriekulturane er sendt til Pharmaq analytiq og det er påvist vibriobakteriar (når type vibrio er bestemt vil endeleg prøvesvar bli*

> *ettersendt) ..... Det er truleg at infeksjon av vibriobakteriar / utvikling av sår og sepsis er årsak til dødelegheit. Det er ikkje truleg at infeksjon med costia [Ichthyobodo sp.] har betydning for dødelegheita. Det er ikkje mistanke om annan Sjukdom."*

The Veterinary report describes infection with a *Vibrio* bacterium in 4 dead or moribund individuals in the tank with the 2151 experimental fish of all types (Sjukebesøk July 7th). The salinity in the tank was reduced from 25 to 15 ‰ salt during the Sjukebesøk on July 7 th. No information has been given about the identity (*dnd*-knockout or control) or size of these fish. The relevance of this information regarding the total loss of 26 fish out of the 303 putative *dnd*-KO/*dnd*-KO fish, to VKM's risk assessment remains uncertain. To determine whether this would affect the risk, and thus fall under VKMs mandate for this project, VKM would need to know the type of *Vibrio*, total mortality and whether the infection affected control fish and *dnd*-KO fish equally.

Conclusions

Based on the new data supplied by the applicant, VKM assesses that the likelihood of transferring sterility alleles to wild populations of Atlantic salmon is considerably reduced as the number of experimental *dnd*-KO/*dnd*-KO fish is reduced from a putative 303 individuals to 163. Also, all remaining 163 trial candidates have a confirmed knockout/knockout genotype that has been confirmed to result in sterility by representation of 61 sacrificed fish that were analysed by qPCR and histology.

However, the re-sequencing of 276 individuals also showed genetic differences from the original sequencing, including 24 individuals that showed a new allele and three individuals that changed category (from sterile to likely fertile). Based on this, VKM cannot completely rule out that there are still fertile carriers of a sterility allele among the putatively sterile trial candidates due to genotyping errors. Therefore, as the potential impact (massive) remains the same, the risk concluded in VKM 2023:20 (potentially high risk) still applies, although the likelihood (very unlikely) is even lower now.

VKM originally also considered the risk associated with other ecological effects that could occur if only sterile fish escaped (i.e., predation on juvenile wild salmon and behavioural effects during spawning). The reduction of the research trial to include 163 (instead of 303) *dnd*-KO fish in sea cages reduces the number of individuals that could possibly escape, and thereby also the likelihood of any impact occurring (very unlikely). In VKM Report 2023:20 the risk associated with other ecological effects was characterized to be low, which VKM now upholds. However, the high uncertainty remains as the necessary experiments have not been carried out.

Sammendrag på norsk

Bakgrunn

I 2023 uførte VKM på oppdrag fra Miljødirektoratet en risikovurdering av et feltforsøk med genmodifisert steril VIRGIN® laks (*Salmo salar*) i oppdrettsliknende miljø (merder i sjøen) fra postsmoltstadiet til høsting (VKM Report 2023:20). Søker er Havforskningsinstituttet (HI) og forsøket er planlagt utført ved Havforskningens Forskningsstasjon Matre. Forsøksmaterialet var F1 avkom av F0 foreldre som var injisert med CRISPR/Cas9 for å slå ut et gen (*dnd*) som er nødvendig for dannelsen av kjønnsceller (og derfor genetisk sterile), og som var injisert med mRNA for å opprettholde fertiliteten. 303 *dnd*-knockout (KO)/*dnd*-KO og 485 wildtype (wt)/wt som kontroll ble identifisert ved genotyping av 2200 avkom etter to F0-hanner krysset med fire F0-hunner. VKM konkluderte i sin rapport med at forsøket var forbundet med potensielt høy risiko. Dette fordi VKM vurderte det til at det ikke kunne garanteres (ved kun en runde med genotyping) at det ikke fantes feil-genotypete individer som ved rømming kunne overføre genetisk sterilitet til ville laksebestander.

I vurderingen fra 2023 identifiserte og vurderte VKM aktuelle risikoreduserende tiltak. Ett av tiltakene var analyse av flere representanter fra hver av de vanligste *dnd*-KO-mutasjonene med hensyn til gonadeutvikling (dissekering, histologi og genuttrykk), samt utføre en bekreftende genotyping av alle de 303 *dnd*-KO/*dnd*-KO og alle de 485 wt/wt. Miljødirektoratet har i brev til VKM av 8. april 2024 bedt VKM vurdere i hvilken grad opplysninger og dokumentasjon sendt fra HI 29. februar 2024, samt ytterligere informasjon sendt fra HI 8. mars 2024, endrer VKMs konklusjoner i opprinnelig risikovurdering av feltforsøk med genmodifisert steril laks.

Nye data levert av søker

VKM har vurdert de nye dataene som er levert av søker. Disse dataene omfatter:

1. Re-genotyping av alle individer som var tiltenkt brukt i feltforsøket (n=277)
2. Genuttrykk av *dnd* og *vasa* (n=61)
3. Morfologiske undersøkelser av gonadeutvikling (n=61)
4. Histologiske undersøkelser av gonadevev (n=61)

I tillegg har søker erstattet de tiltenkte wt/wt kontrollfiskene med konvensjonell oppdrettslaks.

VKM, søker og relevante eksperter hadde også et møte 15. april for å oppklare spørsmål knyttet til de innsendte dataene. I dette møtet etterlyste VKM informasjon for å belyse omstendighetene rundt reduksjonen av potensielle forsøksfisk fra 303 til 277.

VKMs vurdering av de nye dataene

VKM har vurdert dokumentasjonen av 61 åpnete *dnd*-KO/*dnd*-KO individer og mener det er dokumentert at disse viser histologi og genuttrykk som samsvarer med at de er sterile. VKM har også vurdert HI sin bekreftende genotyping av 276 (av de 303 *dnd*-KO/*dnd*-KO) individer. Med en blanding av MiSeq og Sanger-sekvensering ble det identifisert 247 individer med korrekt dobbelt genotyping, 24 med ny mutasjon, ins22, og 3 med feil svar (deriblant én med *dnd*-KO/wt og gonadeutvikling, en annen med *dnd*-KO/sub [= substitusjon] og en tredje med wt/wt). Av de 247 individene ble 61 åpnet for histologi og genuttrykk og én ble funnet død. Av de gjenværende 185 har 22 *dnd*-KO-mutasjoner som ikke er representert blant de 61 som det er utført histologi på. Dette gir 163 *dnd*-KO/*dnd*-KO med en betydelig større sikkerhet for å være sterile enn de 303 opprinnelige.

Ytterligere vurderinger og betraktninger

VKM har ikke mottatt entydige opplysninger om hvorfor 277 og ikke 303 *dnd*-KO/*dnd*-KO ble valgt ut til bekreftende genotyping (ett finneklipp ble mistet). Differansen på 26 individer skjedde ved økt dødelighet to uker etter at forsøksfiskene ble satt på 25 promille sjøvann sommeren 2023. Det er ikke kjent om dødeligheten skyldtes de-smoltifisering og manglende sjøvannstoleranse, eller også *Vibrio*-bakterier som ble påvist i sjukebesøk 7. juli.

Opplysninger om dødelighet knyttet til infeksjon med *Vibrio* er opplysninger som VKM mottok rett før leveringsfristen for dette oppdraget. Grunnet mangel på data om hvilken type *Vibrio*-bakterie som ble oppdaget, samt hvor vidt forsøksfisken og kontrollfiskene ble påvirket på lik linje, er det uavklart om denne dødeligheten burde vært inkludert i risikovurderingen.

Konklusjoner

VKM finner at det på bakgrunn av de nye dataene er betydelig lavere usikkerhet knyttet til hvorvidt mutasjonene som er inkludert i de histologiske undersøkelsene medfører fenotypisk sterilitet. Med tanke på potensielle negative effekter gjennom genetisk påvirkning av ville laksefisk (massiv), finner likevel VKM at den samme risikokategorien (potensielt høy risiko) fortsatt gjelder, selv om sannsynligheten (veldig usannsynlig) for negative effekter nå er enda lavere enn tidligere. Bakgrunnen for å opprettholde denne konklusjonen er knyttet til usikkerhet om hvor vidt det likevel kan være fisk med andre genotyper i forsøksmaterialet. Dette bunner i at tre individer hadde feil genotype og at 24 hadde en ny *dnd*-mutasjon ved andre gangs genotyping. Dette tyder på at genotypingen av forsøksfiskene er forbundet med høy feilrate. Det kan ikke derfor utelukkes at det finnes individer blant de gjenværende 163 som er fertile bærere av et sterilitetsallel.

I den opprinnelige risikovurderingen konkluderte VKM med at det er lav risiko for andre økologiske effekter (knyttet til predasjon og adferd). Det er ikke kommet inn nye opplysninger, utover at antallet fisk reduseres fra 303 til 163. Konklusjonen om lav risiko opprettholdes derfor. Usikkerheten knyttet til eventuelle effekter er imidlertid fortsatt høy. Denne usikkerheten kan reduseres ved forsøk i lukkede tanker på land.