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VKM Report 2023:20

Environmental risk assessment of genetically modified sterile VIRGIN® Atlantic salmon for use in research trials in aquaculture sea-cages

Kjetil Hindar, Johanna Bodin, Nur Duale, Anne-Marthe Ganes Jevnaker, Åse Helen Garseth, Martin Malmstrøm, Kristian Prydz, Ville Erling Sipinen, Eva B. Thorstad, Paul Ragnar Berg, Knut Tomas Dalen, Tor Atle Mo, Ingrid Olesen, Espen Rimstad and Gaute Velle

Scientific Opinion

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Preparation of the opinion

The Norwegian Scientific Committee for Food and Environment (Vitenskapskomiteen for mat og miljø, VKM) appointed a project group to draft the opinion. The project group consisted of five VKM members, three VKM staff and one external experts. An interdisciplinary VKM approval group appointed specifically for the assignment, assessed, and approved the final opinion.

Authors of the opinion

The authors have contributed to the opinion in a way that fulfils the authorship principles of VKM (VKM, 2019). The principles reflect the collaborative nature of the work, and the authors have contributed as members of the project group and/or the VKM Panel on … /the VKM Scientific Steering Committee/an interdisciplinary VKM approval group, appointed specifically for the assignment.

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Competence of VKM experts

Persons working for VKM, either as appointed members of the Committee or as external experts, do this by virtue of their scientific expertise, not as representatives for their employers or third-party interests. The Civil Services Act instructions on legal competence apply for all work prepared by VKM.

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Summary

Background

One of the substantial environmental challenges posed by the aquaculture industry is the escape of farmed Atlantic salmon (*Salmo salar*), which can mate with wild Atlantic salmon and alter the genetic composition of the wild populations. One potential solution to mitigate this issue is the cultivation of sterile salmon in aquaculture. Atlantic salmon can be made sterile by pressure or temperature treatment of newly fertilized eggs to produce triploids, which are functionally sterile due to their unpaired chromosomes. However, these triploids often perform poorly on commercial fish farms and the production of triploid salmon in Norway is put on hold due to welfare issues of the fish.

In this application, the Institute of Marine Research (IMR), Bergen, seeks to rear genetically modified sterile Atlantic salmon (VIRGIN® salmon) in a marine aquaculture environment from the post-smolt stage until harvest. The research trials are to take place in small, open sea cages (net pens) at the IMR Matre Aquaculture Research Station from autumn 2023 until February 2025. The Norwegian Environment Agency has asked VKM to assess the environmental risks associated with this field trial according to the Gene Technology Act and using risk assessment guidance from the European Food Safety Authority, EFSA.

The research trial material and rearing environment

The Atlantic salmon used in the experiment are offspring of parental fish (F0 generation) produced by a precise gene editing method (microinjection of CRISPR-Cas9 at the egg stage) introducing various point mutations leading to a non-functional gene sequence of the dead end (*dnd)* gene. This *dnd* gene is essential for germinal cell development since non-functional gene sequences prevent the development of gonads and lead to genetic sterility. To produce fertile parental fish, a stabilized mRNA encoding a functional dnd protein was injected together with the CRISPR-Cas9 constructs. This approach results in genetically sterile parental fish (functional knockout of the *dnd* gene), while they remain physiologically fertile due to translation of the functional dnd protein, which promotes the development of gonad tissue. These fish are referred to as F0 VIRGIN rescued salmon. In November 2021 two males and four females of the F0 VIRGIN rescued salmon were crossed, and a total of 2200 offspring (F1 generation) was produced. Of these 2200 offspring, 303 genetically sterile fish (F1 VIRGIN salmon) and 485 wildtype fish as control, were selected for the experiment based on their genotype in adipose fin-clips. The 303 experimental fish show genotypes that carry two *dnd* non-functional gene variants (alleles), whereas the control fish carry two functional wildtype alleles. The mosaic nature (not all cells have been mutated) of the parental F0 fish resulting from CRISPR-Cas9 editing enables the presence of both sterile individuals (F1 VIRGIN salmon) and fertile individuals (wildtype farmed salmon) within the same sibling offspring groups.

The research trials will be carried out in sea cages (open net pens) at the Smørdalen fish-farming facility (locality no. 12154) at Matre Aquaculture Research Station. All 788 individuals are PIT-tagged, vaccinated and length-measured and weighed prior to release into the sea cages at approximately 400 g average weight. Double net pens are used with four 5 x 5 m net pens (5 m deep), situated within a single 12 x 12 m net pen (14 m deep) and covered by double netting. Each of the 5 x 5 m nets will receive 197 (788/4) individuals that are monitored by PIT antennas. The fish will be sampled

after 5 months, 10 months and at slaughter when the fish weigh approximately 5 kg. Sampling includes measurement of length and weight and recording of welfare indicators, such as malformations, injuries, and other signs of health issues. The experimental fish will be inspected daily, and if there are any dead fish, they will be collected. Environmental conditions like conductivity, temperature, and depth profiling will be recorded. If there is an escape, the applicant will follow standard procedures for capturing escaped farmed salmon. The study facility has access control, and the sea cages are marked with GMO labelling. However, the PIT tags used are small and internal, so there is no external marking to identify the fish as GMO. Upon detection of disease, the experiment will be terminated. If all fish survive until slaughter, a total of approximately 1515 kg of experimental fish will be incinerated (303 individuals at 5 kg).

Methodology and data

The VKM project group for this risk assessment has expertise in molecular biology, biochemistry and toxicology, Atlantic salmon genetics and ecology, fish diseases, and interactions between fish farming and wild salmonids, including escaped farmed salmon. VKM also appointed an approval group with expertise in CRISPR-Cas9, aquaculture genetics, fish genomics, aquatic ecosystems, parasitology, and virology.

VKM has assessed the application according to national and international (EFSA) guidelines for environmental risk assessment of genetically modified organisms. VKM has put emphasis on identifying knowledge gaps and uncertainties in the risk assessment of the research trial.

Molecular characterization

VKM finds that the data provided in the application are not sufficient to confirm that the genotypes of all 303 homozygous (mut/mut) F1 VIRGIN salmon and all 485 homozygous (WT/WT) fish are correct for all individuals.

Furthermore, VKM finds that it is not sufficiently documented that all genotypes identified among the 303 F1 VIRGIN salmon indeed result in germ cell free and/or sterile individuals. Some of the double allelic mutations identified among the 303 F1 VIRGIN salmon are assumed by the applicant to result in germ cell free sterile fish, but this assumption is not supported by confirmative experimental data in the application.

Overall, based on the information provided in the application, VKM concludes that there is a possibility that an unknown number of potentially fertile individuals of F1 VIRGIN salmon with allelic mutation(s) in the *dnd* gene are erroneously registered as double allelic mutants (n = 303). Likewise, an unknown number of the homozygous wildtype controls (n = 485) may in fact be fertile heterozygous fish, carrying a mutated *dnd* allele.

Environmental risk assessment

No experiments have been carried out in contained facilities that could inform the environmental risk assessment (ERA) for this application:

- Crosses between F0 VIRGIN rescued or F1 VIRGIN salmon and wild salmon have to our knowledge not been carried out for studies of behaviour, reproduction, and offspring

survival. Such crosses could be carried out in contained facilities and informed the assessment of possible risk to wild salmon in cases where potentially fertile fish escape.

- The feeding behaviour of F1 VIRGIN salmon has to our knowledge not been studied in contained facilities. This could have informed the risk assessment with respect to feeding behaviour in sea water and fresh water, especially with respect to potential predation on juvenile salmonids.

- Neither a targeted research design nor laboratory methods have so far been applied to investigate potential differences in immunocompetence and susceptibility to infectious agents between F1 VIRGIN salmon and the wildtype controls.

Uncertainties and data gaps

Correspondence between the *dnd* knockout genotype in adipose fin-clips and the germ cell-free phenotype in F0 VIRGIN rescued salmon has been shown in a limited number of individuals and discrepancy has been reported due to mosaicism.

Information on a confirmative second genotyping of all individuals included in the study, both wildtype (485) and fish with double knockout alleles (303), is missing. Undeliberate errors may have been introduced in the analysis of 2200 samples due to sample quality, sample handling, selection and marking of the individuals to be included in the study. Confirming the data is essential for the determination of the genetical background included in the study.

Limited information is available about the less common mutations included in the study, since the presented data confirming sterility only covers the most common mutations. About 7% of the *dnd* mutations represented in the 303 F1 VIRGIN salmon (n=20) are less well-characterized genotypes, where lack of gonad development has not been confirmed by dissection, i.e., morphological, or histological examination.

The likelihood of genetic confounding increases when only two males by four females are used to produce the 303 F1 VIRGIN salmon and their 485 wildtype sibling controls. In addition, information about family representation among the fish in the different experimental groups is missing. This may introduce biased estimates of effects and hence reduce the validity of the results.

The experimental design does not follow EFSA (2013) guidelines on choice of comparators in situations where escape from captivity is possible.

The lack of external marks on the experimental fish (PIT-tagged F1 VIRGIN salmon and wildtype siblings) adds uncertainty as to where experimental fish might spread if escaping from the facility at Matre.

No plan on how to study the response of experimental fish to infectious agents, that are likely to be introduced to the research material within the planned 1.5 years in net pens, has been presented.

Assessment of hazards

VKM assesses that it is uncertain whether the likelihood of fish escaping from the experimental net pens differs from the likelihood of escaping from standard net pens used in fish farming. On the one hand, small net pens anchored within a larger net pen may better contain fish during most operations, and on the other, this system may perform less well during periods with extreme weather conditions.

Hazards identified for this research trial include the potential fertility of some of the presumedly sterile fish generated for the experiment. The provided information regarding methodology and molecular characterization does not fully support the assumption that all genotypes of the 303 experimental fish with double allelic mutations lead to sterility, particularly, those with less common mutation types.

If heterozygote fish (with one fertility allele and one sterility allele in the *dnd* gene) escape, they could introduce the sterility allele into wild salmon populations by vertical gene transfer to the offspring generation and to the following generations. The spread of the sterility allele would go unnoticed as it would be recessive in heterozygote individuals. The recessive homozygote offspring (two sterility alleles) of heterozygote pairs would compete for food and space in rivers as juveniles and thereby reduce population productivity and reduce the viability in vulnerable populations. VKM assesses this as a potentially high risk.

Another hazard considered by VKM is that escaped sterile individuals from the experiment could enter rivers at a larger size than immature conventional farmed salmon, and act as predators on juveniles of wild Atlantic salmon and brown trout (*Salmo trutta*). It is known that conventional farmed salmon may enter rivers as immature as well as sexually mature individuals, with immature escapees typically smaller than mature escapees. Sterile large-sized individuals could therefore constitute an ecological novelty in rivers if they acted predators. VKM considers this risk to be low. The risk is associated with a high uncertainty, as the necessary experiments have not been carried out.

The status of wild Atlantic salmon populations is poor in the region (Vestland county), where Matre is situated, according to the Quality Standard for wild Atlantic salmon populations. The pressure from aquaculture on wild Atlantic salmon and sea-run brown trout in the same region (production zone PO4) is high. This suggests that escaped salmon from the research trials can decrease the viability of recipient wild populations in the region.

The typically wide dispersal of escaped farmed salmon and the absence of external tags distinguishing them from ordinary farmed escapes, imply that salmon populations can be (unknowingly) affected even when located far away from Matre.

The hazards identified in relation to pathogens, infections and diseases are that F1 VIRGIN salmon get infected and spread infectious agents when held in confined aquaculture (open net pens) at Matre or after escape from confined aquaculture at Matre. In line with the comparative approach of the ERA, VKM assessed whether F1 VIRGIN salmon have a more negative outcome for the environment than the comparators would have had.

Conclusions

A thorough molecular characterization of the fish intended for release into sea cages is needed to fully assess the potential risks to the environment in case of accidental escape. This characterization should include confirmational genotype analysis of all individuals included in the experiment, and confirmation that each unique mutated *dnd* allele present among the experimental fish leads to loss-of-function.

VKM assesses that the research trial on releasing VIRGIN salmon into sea cages from autumn 2023 to February 2025 is associated with potentially high risk for wild Atlantic salmon populations. This is mainly based on two concerning elements. First and foremost, it is based on the information provided in the application, VKM concludes that there is insufficient documentation on proof of sterility in all the 303 F1 VIRGIN individuals and a possibility that an unknown number of potentially fertile individuals of F1 VIRGIN salmon with allelic mutation(s) in the *dnd* gene are erroneously registered as double allelic mutants (n = 303). Likewise, an unknown number of the homozygous wildtype controls (n = 485) may in fact be fertile heterozygous fish, carrying a mutated *dnd* allele.

Secondly, VKM assesses that it is uncertain whether the likelihood of fish escaping from the experimental pens differs from the likelihood of escaping from standard net pens used in fish farming.

Escaped experimental salmon, should they be fertile carriers of mutated *dnd* alleles, may spawn with wild Atlantic salmon. If such salmon are carrying a sterility allele, they may introduce sterility alleles to wild Atlantic salmon populations by vertical gene transfer. The sterility alleles will be recessive and therefore hidden to purging in wild populations, except when in the homozygote state in the second and later generations. If interbreeding occurs, it may reduce the productivity of wild populations and reduce the viability of already vulnerable populations.

VKM assesses that spread of sterility alleles to future generations of wild salmon populations would be a massive impact on wild Atlantic salmon. The overall likelihood of such spread, including salmon escaping from the experiment, is assessed as very unlikely. However, sterility alleles can be introduced to wild populations by very few individuals. VKM therefore concludes that the experiment poses a potentially high risk to wild salmon populations.

VKM assesses that both the magnitude of impact and the likelihood of impact with regards to spread of infectious agents are affected by the number of F1 VIRGIN salmon and wildtype siblings in the experiment. Based on a comparative approach, the environmental risk with regards to spread of infectious agents from release of 303 F1 VIRGIN salmon is assessed to be low, both if F1 VIRGIN salmon are contained at Matre, and if F1 VIRGIN salmon escape. VKM assesses the level of uncertainty to be high in both assessments since available information on the topic is limited, and mostly expert judgements have been used.

Several of the knowledge gaps identified by VKM can be filled by conducting studies in contained facilities. This would reduce uncertainties related to the risk assessment but would not necessarily reduce the likelihood of unwanted effects.

Key words: Atlantic salmon, aquaculture, GMO, genetically modified, dead end, dnd, allelic mutation, environmental impact.

Sammendrag på norsk

Bakgrunn

En av de store miljøutfordringene fra akvakultur er at oppdrettslaks rømmer og krysser seg med villaks, og derved medfører genetiske endringer hos villaksen. En mulighet for å unngå dette er å basere lakseoppdrett på steril fisk. Laks kan gjøres steril ved å gi trykk- eller temperatursjokk til nylig befruktede egg. Dette gir triploide avkom med tre kromosomsett, som er funksjonelt sterile på grunn av ikke-parete kromosomer. Triploide oppdrettslaks har imidlertid vist seg å fungere dårlig i kommersielt oppdrett og videre produksjon av triploid oppdrettslaks i Norge er satt på vent på grunn av dårlig velferd.

I denne søknaden ønsker Havforskningsinstituttet, Bergen, å drette opp genmodifisert, sterile laks (VIRGIN laks) i oppdrettsliknende miljø fra postsmoltstadiet til høsting. Forsøket er planlagt utført i små merder ved Havforskningens Forskningsstasjon Matre fra høsten 2023 til februar 2025. Miljødirektoratet har bedt Vitenskapskomitéen for mat og miljø (VKM) om å foreta en miljørisikovurdering av forsøksutsettingen i henhold til genteknologiloven og ved å bruke retningslinjer utarbeidet av Den Europeiske myndighet for næringsmiddeltrygghet (European Food Safety Authority, EFSA).

Forsøksmateriale og oppdrettsmiljø

Laksen som brukes i forsøket er avkom av oppdrettslaks (F0-generasjonen) produsert med en presis genredigeringsmetode (mikroinjeksjon av CRISPR-Cas9 på eggstadiet), som resulterer i individer med ulike punktmutasjoner, og dermed en ikke-funksjonell gensekvens, i et gen som kalles 'dead end' (*dnd*). Dette genet er nødvendig for dannelse av kjønnsceller, og ikke-funksjonelle gensekvenser hindrer gonadeutvikling og medfører sterilitet. For å sikre fertil foreldregenerasjon blir mRNA som koder for funksjonelt dnd protein mikroinjisert sammen med CRISPR-Cas9. Foreldregenerasjonen er derved gjort genetisk sterile ('knockout' av *dnd* genet) men samtidig gjort fysiologisk fertile ved translasjon av funksjonelt dnd protein som fører til utvikling av gonader. Disse fiskene refereres til som F0 VIRGIN gjenopprettet laks. I november 2021 ble to hanner og fire hunner av disse F0 VIRGIN gjenopprettede laksene krysset, og totalt ble det produsert 2200 avkom (F1 generasjonen). Av disse 2200 ble det valgt ut 303 genetisk sterile (F1 VIRGIN laks) og 485 villtype avkom som kontroll til eksperimentet det søkes om; alle valgt på bakgrunn av genotyping av fettfinneklipp. De 303 forsøksfiskene viser genotyper med to muterte genvarianter (alleler) av *dnd*, mens de 485 kontrollfiskene har to funksjonelle villtypealleler. Den mosaiske naturen (ikke alle cellene har fått indusert mutasjonen) til F0-fiskene redigert med CRISPR-Cas9 gjør det mulig å finne både sterile individer (F1 VIRGIN laks) og fertile individer (villtype oppdrettslaks) blant avkom i de samme søskengruppene.

Forsøket skal utføres i merder på Smørdalen sjøanlegg (lokalitet nr. 12154) på Forskningsstasjon Matre. Alle de 788 individene er PIT-merket, vaksinert, lengdemålt og veid før de skal settes ut i merder ved en gjennomsnittlig kroppsstørrelse på om lag 400 gram. Det skal brukes doble nøter der fire 5 x 5 meter merder (5 m dype) er plassert inni en 12 x 12 m merd (14 m dyp) som har dobbel netting over. Hver 5 x 5 m merd skal motta 197 (788/4) individer som overvåkes med PIT-antenner. Det skal tas prøver av fiskene etter 5 måneder, 10 måneder og ved slakting når fisken veier om lag 5 kg. Prøvetakingen inkluderer måling av lengde og vekt og registrering av velferdsindikatorer slik som misdannelser, sår og andre tegn til svak helsestatus. Død fisk skal samles inn daglig og miljøvariable som ledningsevne og temperatur ved ulike dyp registreres automatisk. Dersom det skjer en rømming,

vil søkeren følge standard prosedyre for gjenfangst av rømt oppdrettslaks. Oppdrettslokaliteten har adgangskontroll og merdene er utstyrt med GMO-merking. Imidlertid er PIT-merkene som brukes til å merke fisken små, indre merker, så selve fisken har ikke noen ytre merking som viser at de er GMO-fisk. Ved eventuelle sykdomsutbrudd vil forsøket bli avsluttet. Dersom all forsøksfisk overlever fram til slakting, blir totalt om lag 1515 kg forsøksfisk destruert (303 individer a 5 kg).

Metodikk og data

VKMs prosjektgruppe for denne risikovurderingen har ekspertise i molekylærbiologi, biokjemi og toksikologi, laksens genetikk og økologi, fiskesykdommer og interaksjoner mellom akvakultur og ville laksefisk, inklusive rømt oppdrettslaks. VKM oppnevnte også en godkjenningsgruppe med ekspertise i CRISPR-Cas9, akvakulturgenetikk, genomikk, akvatiske økosystemer, parasittologi og virologi.

VKM har vurdert søknaden i henhold til nasjonale og internasjonale (EFSA) retningslinjer for risikovurdering av genmodifiserte organismer. VKM har lagt vekt på å identifisere kunnskapshull og usikkerhet i sin vurdering av forsøket.

Molekylær karakterisering

VKM finner at dataene som er gitt i søknaden ikke er tilstrekkelige til å bekrefte at genotypene til alle 303 homozygote (to muterte alleler) F1 VIRGIN laks og alle 485 homozygote (to villtypealleler) oppdrettslakskontroller er korrekt bestemt for alle individer.

Videre finner VKM at det ikke er tilstrekkelig dokumentert at alle genotypene funnet blant de 303 F1 VIRGIN laks faktisk resulterer i kjønnscelleløse og/eller sterile individer. Noen av de doble alleliske mutasjonene identifisert blant 303 F1 VIRGIN laks er antatt av søker å resultere i kjønnscellefrie sterile laks, men denne antagelsen er ikke støttet av bekreftende eksperimentelle data i søknaden.

Basert på informasjonen gitt i søknaden, konkluderer VKM at det er en mulighet for at et ukjent antall potensielt fertile individer blant F1 VIRGIN laks med allelisk(e) mutasjoner i *dnd* genet feilaktig er registrert som doble alleliske mutanter (n=303). På samme måte kan et ukjent antall individer blant homozygote villtypekontroller (n=485) være fertile heterozygote laks med et mutert *dnd* allel.

Miljørisikovurdering

Det er ikke utført eksperimenter i lukkete fasiliteter som kunne ha gitt informasjon til miljørisikovurderingen, herunder:

- Krysninger mellom F0-gjenopprettede eller F1 VIRGIN laks og villaks er ikke studert med hensyn til atferd, reproduksjon eller overlevelse. Slike krysninger kunne vært utført i lukkete anlegg og gitt data til mulig risiko for villaks i tilfeller der potensielt fertil fisk har rømt.
- Spiseatferd til F1 VIRGIN laks har så vidt vi vet ikke vært studert i lukkete anlegg. Dette kunne gitt informasjon til risikovurderingen med hensyn til spiseatferd i sjøen og i ferskvann, spesielt i forhold til mulig predasjon på laks- og ørretunger.
- Undersøkelser av mulige forskjeller i immunkompetanse og mottagelighet for smittsomme agens mellom F1 VIRGIN laks og villtypekontroll er så langt verken muliggjort av forsøksdesign eller laboratoriemetoder.

Usikkerhet og kunnskapshull

Korrespondanse mellom *dnd* knockout-genotype i fettfinne og kjønnscellefri fenotype er vist i et begrenset antall individer av F0 VIRGIN gjenopprettede laks og det er rapportert uoverensstemmelser som skyldes mosaisme.

Det mangler informasjon om hvorvidt det er gjennomført bekreftende genotyping av fiskene som skal brukes i forsøket, både villtype (485) og de med doble knockout-alleler (303). Analysen av genotypene til 2200 prøver kan medføre en viss risiko for feilbestemming forårsaket av prøvekvalitet, prøvehåndtering, seleksjon og merking av individene. Tillit til resultatene av genotypingen er avgjørende for å være sikker på den genetiske sammensetningen som inngår i forsøket.

Det er begrenset informasjon om de mindre vanlige mutasjonene siden de bekreftende dataene for sterilitet kun dekker de vanligste mutasjonene. Om lag 7% av *dnd*-mutasjonene som er representert blant 303 F1 VIRGIN laksen (n=20) har mindre velkarakteriserte genotyper, der mangel på gonadeutvikling ikke er bekreftet gjennom disseksjon i form av morfologiske eller histologiske undersøkelser.

Kun to hanner og fire hunner er blitt brukt til å produsere de 303 F1-VIRGIN laksene og deres 485 villtype avkom. I tillegg mangler informasjon om i hvilken grad de ulike familiene er representert i de ulike eksperimentgruppene. Dette kan introdusere skjevheter i de estimerte effektene og derved redusere validiteten til resultatene.

Eksperimentoppsettet følger ikke EFSA (2013) sine retningslinjer for valg av komparator i forsøksutsettinger der rømming fra utsettingslokaliteten er mulig.

Mangel på et ytre merke som identifiserer forsøksfisken øker usikkerheten i forhold til hvor forsøksfisken (PIT-merket F1 VIRGIN laks og villtypesøsken) kan ende opp dersom de rømmer fra fasilitetene på Matre.

Det er ikke fremlagt noen plan for hvordan man skal undersøke forsøksfiskens respons på infeksiøse agens som sannsynligvis vil bli introdusert i anlegget i løpet av det halvannet året som forsøket er planlagt for.

Farevurdering

Forsøksmiljøet med fire små merder inni en større merd gir ikke nødvendigvis noen reduksjon i den (beskjedne) rømmingssannsynligheten. Oppsettet med doble nøter som er forankret i hverandre vil antakelig fungere bra under normale forhold, men kan fungere dårlig under ekstreme værforhold.

Farer som er identifisert i denne risikovurderingen inkluderer muligheten for at noen av de antatt sterile individene er fertile. Den mottatte informasjonen om metodikk og molekylær karakterisering gir ikke full støtte til antagelsen om at alle genotypene hos de 303 eksperimentelle fiskene med to muterte alleler medfører sterilitet, spesielt for de mindre vanlige mutasjonene.

Dersom heterozygote individer (med ett fertilitetsallel og ett sterilitetsallel i *dnd*-genet) rømmer fra forsøket, kan de introdusere sterilitetsallelet til ville laksebestander gjennom vertikal genoverføring til avkomsgenerasjonen og til påfølgende generasjoner. Spredning av sterilitetsallelet ville være vanskelig å oppdage siden allelet er recessivt og skjult i heterozygoter. Recessive homozygot avkom (med to sterilitetsalleler) etter heterozygote par vil konkurrere om mat og plass i elva som ungfisk, og derved redusere bestandenes produktivitet og redusere de sårbare bestandenes levedyktighet. VKM vurderer dette som en potensielt høy risiko.

En annen potensiell fare som er vurdert av VKM er at rømte sterile individer fra eksperimentet kan gå opp i elv ved en større størrelse enn umoden konvensjonell oppdrettslaks, og være predatorer på ungfisk av villaks og ørret (*Salmo trutta*). Det er kjent at konvensjonell oppdrettslaks kan gå opp i elv både som umodne og kjønnsmodne, der umodne rømlinger vanligvis er mindre enn kjønnsmodne rømlinger. Sterile og store individer i elv kunne derfor ha en annen økologisk effekt enn det vi kjenner fra umodne rømte oppdrettslaks, dersom de er predatorer på ungfisk. VKM anser denne risikoen for å være lav med stor usikkerhet siden relevante forsøk mangler.

Bestandsstatus for laks er dårlig i regionen (Vestland fylke) der Matre er lokalisert, vurdert ut fra Kvalitetsnormen for ville laksebestander. Presset fra akvakultur på villaks og sjøørret i den samme regionen (produksjonssone PO4) er høy. Dette betyr at ytterligere negativ påvirkning fra forsøksfisken på ville bestander i området kan redusere villfiskbestanders levedyktighet.

Rømt oppdrettslaks har vist seg å kunne spre seg langt fra der de rømmer. Siden forsøksfisken det søkes om ikke har et ytre merke som skiller dem fra vanlig oppdrettslaks, kan dette medføre at laksebestander langt unna kan bli påvirket uten at dette er kjent.

Farene som er identifisert i forhold til spredning av smittestoffer er at F1 VIRGIN laks kan bli infisert og spre smittestoffer når den holdes i åpne merder ved Matre eller etter rømming fra åpne merder ved Matre. I tråd med den komparative tilnærmingen til ERA, vurderte VKM om F1 VIRGIN laks har et mer negativt utfall for miljøet enn komparatorene ville hatt.

Konklusjoner

En grundig molekylær karakterisering av forsøksfisken som er ment å settes ut i merd, er nødvendig for å vurdere miljørisiko i tilfelle forsøksfisken skulle rømme. En slik karakterisering burde inkludert bekreftende genotyping av alle individer i forsøket, og en bekreftelse av at alle *dnd* mutasjonene som er inkludert i forsøket gir ikke-funksjonelle alleler.

VKM vurderer at utsettingsforsøket med VIRGIN laks i merd fra høsten 2023 til februar 2025 er forbundet med potensielt høy risiko for ville laksebestander. Dette er basert på to forhold. For det første er det, basert på informasjonen gitt i søknaden, en mulighet for at et ukjent antall potensielt fertile individer blant F1 VIRGIN laks med allelisk(e) mutasjoner i *dnd*-genet feilaktig er registrert som doble alleliske mutanter (n=303). På samme måte kan et ukjent antall individer blant homozygote villtypekontroller (n=485) være fertile heterozygote laks med et mutert *dnd*-allel.

For det andre vurderer VKM det slik at forsøksmerdene ikke nødvendigvis gir noen ekstra sikkerhet mot rømming enn det som er tilfellet i standard oppdrettsmerder.

Rømming fra forsøket kan medføre at et antall potensielt fertile forsøkslaks kan gyte med villaks. Dersom de er bærere av et allel for sterilitet, kan de spre sterilitet til ville laksebestander gjennom vertikal genoverføring. Sterilitetsallelet vil være recessivt og derfor beskyttet mot å bli selektert vekk fra bestanden unntatt i homozygot tilstand etter parring mellom heterozygote bærere. Recessive homozygote avkom (med to sterilitetsalleler) vil konkurrere om mat og plass i elva som ungfisk, og derved redusere bestandenes produktivitet og redusere de sårbare bestandenes levedyktighet.

VKM vurderer at spredning av sterilitetsalleler til kommende villaksgenerasjoner må anses som en massiv negativ påvirkning på villaks. Totalt sett anser VKM sannsynligheten for at dette skjer, inkludert rømning fra anlegget, som svært usannsynlig. Imidlertid kan slik spredning av sterilitetsalleler

forekomme selv med kun et fåtall fisk. VKM konkluderer derfor med at forsøket utgjør en potensielt høy risiko for ville laksebestander.

VKM vurderer at både omfanget og sannsynligheten med hensyn til spredning av smittestoffer påvirkes av antall F1 VIRGIN laks og villtypesøsken i forsøket. Basert på en komparativ tilnærming vurderes miljørisikoen med hensyn til spredning av smittestoffer ved utsetting av 303 F1 VIRGIN laks å være lav, både ved hold av F1 VIRGIN laks i forsøksanlegg ved Matre, og hvis F1 VIRGIN laks rømmer. Nivå av usikkerhet i begge vurderinger er høyt siden tilgjengelig informasjon om temaet er begrenset, og det stort sett er benyttet ekspertvurderinger.

Flere av kunnskapshullene som er identifisert av VKM kan fylles ved å utføre forsøk i lukkete anlegg. Informasjon fra slike forsøk ville redusert usikkerheten knyttet til denne risikovurderingen, men ikke nødvendigvis redusert sannsynligheten for uønskede effekter.

Nøkkelord: Atlantisk laks, akvakultur, GMO, genmodifisert, Dead end-gen, allelisk mutasjon, miljøpåvirkning.

Abbreviations and glossary

Abbreviations

BLAST	Basic Local Alignment Search Tool.
DNA	Deoxyribonucleic acid.
DHA	Docosahexaenoic acid.
EFSA	European Food Safety Authority.
FASTA	Fast Alignment Search Tool – All.
GM	Genetically modified.
GMO	Genetically modified organism.
ISA	ISA (Infectious Salmon Anemia)
LC-MRM-MS	Liquid chromatography/multiple reaction monitoring/mass spectrometry.
mRNA	messenger RNA
OECD	Organisation for Economic Co-operation and Development.
ORF	Open reading frame.
PCR	Polymerase chain reaction.
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
T-DNA	Transfer DNA.
VKM	Norwegian Scientific Committee for Food and Environment
GCF	Germ cell free
RNA	Ribonucleic acid
WT	Wild type

Glossary

Allele	An allele is one of two or more versions of DNA sequence (gene) at a given genomic location. An individual inherits two alleles, one from each parent. If the two alleles are the same, the individual is homozygous for that allele. If the alleles are different, the individual is heterozygous. Naturally occurring functional alleles are called wild-type alleles (WT), while abnormal and often non-functional alleles are called mutants (mt).
Broodstock	Or brood fish, are the parent fish from which fry and fingerlings are produced. The success of stocking programs, fish farms and aquaculture industries depend upon a reliable supply of healthy fry/fingerlings that have a sound genetic base.
Cas9	A CRISPR-associated (Cas) endonuclease, or enzyme, that acts as molecular scissors to cut DNA at a location specified by a guide RNA (gRNA).
Comparator	The unmodified conventional counterpart used as control to the genetically modified organism, to detect differences in characteristics as a result from the genetic modifications.
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats of genetic information. Some bacterial species use such repeats as part of their antiviral response.
CRISPR-Cas9	Molecular machinery enabling gene/genome edits by precisely cutting DNA and then letting natural DNA repair processes to take over. The system consists of the Cas9 enzyme and a guide RNA. CRISPR-Cas9 is used as a molecular tool to change single nucleotides, introduce short insertions/deletions (indels), or insertion of new genes.
Gene knockout	Inactivation of a gene as a result of genetic changes (insertion/deletion/substitution).
Genetically sterile	Loss-of-function mutation in the *dnd* allele causing sterility

Double allelic loss-of-function mutations	An individual who inherits two mutant non-functional allelic versions of a gene, one from each parent, has a non-functional gene, i.e., a double allelic loss-of-function corresponding to a gene knock out
EFSA guidance	Refers to one or several documents published by the European Food Safety Authority (EFSA) that outline specific approaches and considerations for risk assessment.
F0 VIRGIN rescued salmon	Equivalent to "rescued dnd crispants". I.e., parental (F0) male or female genetically sterile salmon made functionally fertile by injecting *dnd* mRNA together with CRISPR-Cas9 machinery into 1-cell stage embryos, facilitating germ cell migration and (mosaic) gonad formation during development.
F1 VIRGIN salmon	Offspring of F0 VIRGIN (rescued) salmon. F1 VIRGIN salmon have inherited double allelic mutation(s) in the *dnd* gene causing a loss-of function and germ cell free (sterile) salmon.
Genetic modification	The process of inserting novel DNA/genes from the same or foreign species or deleting genes. Common to all is the use of recombinant DNA technology.
Genome editing	The process of editing DNA with techniques such as CRISPR to target genetic changes to a specific location in a genome. Most often with the aim to change single nucleotides or produce short insertions/deletions (indels).
Genotype	The sum of all genes/allelic variants in an organism, or may alternatively be used to indicate allelic variants of a single gene.
Germ cells	Embryonic cells with the potential of developing into gametes.
Gonads	Reproductive gland, a mixed gland that produces the gametes and sex hormones of an organism.
Gonad formation	Primordial germ cells migrate to the developing gonads, which will form the ovaries in females and the testes in males. After a period of mitotic proliferation, the primordial germ cells undergo

	meiosis and differentiate into mature gametes—either eggs or sperm.
Guide RNA (gRNA)	A RNA molecule that binds to Cas9 and a specific DNA region. The gRNA specifies, based on the sequence of the gRNA, the location at which the Cas9 will cut double stranded DNA.
Homozygous	Homozygous, as related to genetics, refers to having inherited the same versions (alleles) of a genomic marker (e.g., a gene), from each biological parent.
Heterozygous	Heterozygous, as related to genetics, refers to having inherited different versions (alleles) of a genomic marker (e.g., a gene) from each biological parent.
Mosaic	Mosaicism in genetics is defined as the presence of two or more cell lineages with different genotypes arising from a single zygote in a single individual.
Mutation	A mutation is a change in the nucleotide sequence of DNA that happens during replication prior to cell division. In this document, we also include in the term changes to DNA sequences that are made by gene editing tools like CRISPR-Cas9.
Mutant	Organism or a gene that carries a mutation.
Off-target effects	Unintended effects when DNA is altered at sites in the genome not intentionally targeted.
Open reading frame	Open reading frame, ORFs are roughly defined as spans of DNA sequence between start and stop codons, that encode an amino acid sequence (protein) according to the genetic code.
Phenotype	The composite of an organism's observable characteristics or traits. An organism's phenotype results from two basic factors: the expression of an organism's genetic code, its genotype, and the influence of environmental factors.
Phenotypically fertile	A fish with detectable gonads
Post-market monitoring	A predefined strategy to monitor for possible adverse effects to human or animal health and the environment.

Receiving environment	The environment that interacts with the organism upon release.
Rescued *dnd* crispants	In this report referring to genetically sterile (non-functional *dnd* gene) salmon made functionally fertile by injecting *dnd* mRNA together with CRISPR-Cas9 machinery into 1-cell stage embryos, facilitating germ cell migration and gonad formation during development.
Startfeeding	Startfeeding is the life stage when a juvenile Atlantic salmon is ready to take feed in a hatchery, or emerge from the gravel in nature to search for food.
Smoltification	A complex series of physiological changes where young salmonid fish adapt from living in fresh water to living in seawater.
Smolt	A young salmon about two years old that is at the stage of development when it assumes the silvery color of the adult and is ready to migrate to the sea.
Transgene	A DNA segment (often a gene) that is transferred from an organism of one species to an organism of another species by genetic engineering.
Unintended effect	Predicted or unpredicted effects from the genetic modification occurring at the genetic, organismal and/or environmental level.
Vector	A vehicle, often a virus or a plasmid carrying desired DNA into a host cell which may also assist in multiplying or expressing the insert.

Background as provided by the Norwegian Environment Agency

According to the Gene Technology Act the Norwegian Environment Agency is the competent authority for the deliberate release of genetically modified organisms for research purposes (field trials, experimental release). The Norwegian Environment Agency has received an application for the deliberate release of genetically modified farmed salmon in sea cages. The applicant is the Institute of Marine Research.

About the application

The field experiment will be carried out at the Institute of Marine Research's research station Matre Aquaculture Research Station in Smørdalen. The applicant has stated that the field trial is part of a larger research project stretching for several years. In this part of the project, the applicant will study the welfare and growth of genetically sterile, germ cell free salmon VIRGIN fish in sea cages. The applicant applies to release 303 VIRGIN genetically modified fish, and 485 control fish (not modified) in a field trial lasting around 1.5 years.

Risk assessment of field trials according to the Gene Technology Act

The effect on the environment and public and animal health that may arise from the deliberate release of GMOs through a limited field trial, constitutes an element in the assessment of the application. The Norwegian Environment Agency therefore commissions VKM to assess the risk of releasing genetically modified farmed salmon through a field trial at the aforementioned location.

Appendix 1 of the Regulation relating to impact assessments pursuant to the Gene Technology Act provides the information that the applicant is asked to submit in the application, together with an environmental risk assessment. The information section is divided into a section A which applies to all genetically modified organisms that are not higher plants, these are covered by section B.

There is no requirement to submit a specific subset of the information specified in Annex 1, Part A, if it is not relevant or necessary for the risk assessment for the particular application, in particular with regard to the genetically modified organism's characteristics, the scope of and the conditions for the deliberate release or intended terms of use.

There are five subsets of information in Appendix 1 related to the GMO and its release:

- I General information
- II Information about the genetically modified organism
- III Information on release conditions and the recipient environment
- IV Information on interactions between the genetically modified organisms and the environment
- V Information on plans for monitoring, control, waste treatment and emergency plans

The level of detail for each subset of information may also vary depending on the nature and extent of the proposed release.

Appendix 2 of the Regulation relating to impact assessments pursuant to the Gene Technology Act provides the principles and methodology to be included in the environmental risk assessment. These are based on criteria in the EFSA guidance and are the same as those found in the annexes to the Directive 2001/18 on the deliberate release into the environment of genetically modified organisms (Directive 2001/18/EC). Some of the points in the environmental risk assessment apply only to marketing applications. In this assignment these points about the marketing applications have been removed, so that it should be easier to see which points apply to the applications for field trials.

Legal background:
Act of 2 April 1993 no. 38 relating to the Production and Use of Genetically Modified Organisms, etc. (the Gene Technology Act) regulates the deliberate release into the environment of genetically modified organisms (GMO). Regulation of 16 December 2005 no. 1495 relating to impact assessments pursuant to the Gene Technology Act.

Through the EEA agreement (European Economic Area Agreement), Norway is acceded to the EU's approval system for the release of GMOs into the environment in accordance with Directive 2001/18/EC. The Directive is transposed into Norwegian law through the Gene Technology Act.

Terms of reference as provided by the Norwegian Environment Agency

The Norwegian Environment Agency refers to the current assignment for risk assessment of GMOs, where VKM shall assess health and environmental risks for applications for the marketing of GMOs under Directive 2001/18/EC or Regulation 1829/2003 on genetically modified food and feed.

This assignment concerns risk assessment in the event of a limited deliberate release of GMOs through a field trial. The field experiment with genetically modified farmed salmon will take place at one location which, according to the applicant, is approved for experiments with farmed salmon. When the experiment ends, the experimental animals will be destroyed. The experimental animals will not enter the human food chain. Thus, the assignment does *not* include assessment for use as food and feed.

> Considerations regarding animal welfare is addressed in Norwegian legislation for animal welfare and is not part of this assignment.

An assessment of the possible effects of the specific genetic modification on the health of the genetically modified organism is, on the other hand, part of the health and environmental risk assessment. See supplementary description under point 3.3 of this assignment letter.

VKM will receive the application's Part I (information) and Part II (environmental risk assessment), in addition to the SNIF (summary of the application), for assessment. Further details about the assignment follow below.

1. Assessment of whether information is sufficient or if there is a need for more information:

In order to be able to carry out a risk assessment of the GMO in accordance with the requirements set out in regulations on impact assessment pursuant to Article 15 of the Gene Technology Act, VKM is requested, among other things, to assess the submitted information and documentation from the applicant, formulate questions and point out any deficiencies and the need for further information from the applicant.

Information requirements for the specific parts of the application to be assessed by VKM:

• General information, including information on personnel and training

• Information relating to the genetically modified organism(s) for which approval is sought

• Information relating to the conditions at the time of release and the recipient environment

• Information on the interaction between the genetically modified organism(s) for which approval is sought and the environment

• A monitoring plan with a view to mapping the effects of the GMO on human and animal health, and the environment

• Information on control, remedial methods, waste treatment and emergency plans

• A summary of the technical documentation

In addition, the application must include an environmental risk assessment, of which shall be assessed by VKM. When it comes to data requirements for applications for field trials, the environmental risk assessment must at least be based on already available data from scientific literature or other sources and can be supplemented with additional data provided by the applicant. Any deficiencies and the need for further information must be sent in writing to the Norwegian Environment Agency with a justification.

2. Assessment of confidential information

In applications regarding deliberate release, any information must in principle always be publicly available. At the request of the applicant, the following information can be exempted from access, if it can be documented that access could significantly harm the applicant's interests:

a. Information about the manufacturing or production process, excluding information that is relevant to the security assessment

b. Information on commercial relations between a manufacturer or importer and the applicant or holder of the approval

c. Information showing the applicant's acquisitions, market shares or business strategies

d. information about DNA sequences, excluding sequences used to detect, identify and quantify the transformation event (gene modification event)

e. information on breeding patterns and breeding strategies

If the applicant requests that some of the above information be exempted from public disclosure, VKM must assess what can be included in the public part of the risk assessment report from the application. The risk assessment itself regarding possible effects on human health, animal health or the environment is not exempt from public disclosure.

In this case, the applicant has stated that all information in the submitted application can be processed openly. In first instance, there is therefore no need for VKM to assess a possible request for confidential information. If there is a need for supplementary information and documentation, it will be clarified with the applicant whether this should also be publicly available information.

3. Assessment of risks to the environment, public and animal health that may arise as a result of the field trial

The risk assessment must, among other things, consist of an assessment of:

3.1 Molecular characterization of the modification and the genetically modified organism

3.2 Effects on the environment, including but not limited to:

- Risk of spread, survival and persistence of the GMO in the environment, and further assessment of possible consequences
- Risk of gene flow
- Risk of affecting the animal health of other wild animals
- Influence of abiotic and abiotic processes

3.3 If VKM deems it appropriate: Effects of the genetic modification on the animal health of the fish that may have environmental effects, including:

- Changed/increased susceptibility to disease-causing substances or less susceptibility to veterinary medical treatment (which may promote the spread of infectious diseases, or create new reservoirs or vectors)

3.4 Effects on human health in contact with the GMO (not for food), including, but not limited to:

- Associated pathogens, toxins, etc

3.5 Assessment of the effect of risk-reducing measures proposed in the application

For limited field trials, long-term adverse effects and cumulative long-term adverse effects in the environmental risk assessment shall not be assessed.

For further guidance on specific areas for health and environmental risks of genetically modified fish, reference is made to the EFSA guidance on the ERA for genetically modified animals (with the specification that these apply to marketing applications, and that some parts may therefore not be relevant for a limited field trial), as well as other available literature in the area.

Methodology and Data

Part of the application is the applicant's own risk assessment of the research trial. The Environment Agency has asked that this follows the structure of the Regulation of 16 December 2005 no. 1495 on impact assessment according to the Gene Technology Act. In its correspondence with the applicant, the VKM has asked that relevant points in the EFSA guidelines on risk assessment of genetically modified animals (EFSA 2012, 2013) are considered.

VKM appointed a project group consisting of members of the VKM panels on biodiversity and genetically modified organisms, and one external expert. The VKM project group for this risk assessment has expertise in molecular biology, biochemistry and toxicology, Atlantic salmon genetics and ecology, fish diseases, and interactions between fish farming and wild salmonids, including escaped farmed salmon. VKM also appointed an approval group with expertise in CRISPR-Cas9, aquaculture genetics, fish genomics, aquatic ecosystems, parasitology, and virology.

VKM has assessed details about the experimental material and the methods used from the descriptions provided in the application for the assessment of the molecular characterization. VKM has searched the original publications provided by the applicant for a deeper understanding of details that were not available in the first version of the application.

VKM has used the literature on wild Atlantic salmon and on interactions between aquaculture and wild salmonids in its environmental risk assessment. VKM has also included knowledge from committee reports on the status of wild Atlantic salmon and sea-run brown trout in Norway, and on risks to wild salmonids from aquaculture. VKM has performed various literature searches on the topics discussed in the application but has not performed a systematic literature search for this project.

On three occasions, VKM has requested new information from the applicant on details that were considered necessary for carrying out the risk assessment. On two occasions the Environment Agency implemented a clock stop to the formal handling of the application until answers were received from the applicant. On a third occasion, the questions posed by VKM were answered without a clock stop. VKM has used all information received in its risk assessment. Full timeline of the correspondence with the applicant is listed in Appendix 2.

Introduction

On April 18th 2023 the Norwegian Scientific Committee for Food and Environment (VKM) received a request from the Environment Agency to perform a risk assessment of genetically modified sterile Atlantic salmon (*Salmo salar*) for use in research trials in aquaculture sea-cages. The application to perform the research trials was submitted by the Institute of Marine Research, Bergen. The research trials are intended to take place at the IMR Matre Aquaculture Research Station from September 2023 until February 2025.

The aim of the experiment is to study genetically sterile, diploid Atlantic salmon with respect to their welfare, behaviour and growth in a near-normal aquaculture environment.

The interest in using sterile salmon for aquaculture is long-lasting and stems from the observation that escaped farmed Atlantic salmon enter rivers and interbreed with wild salmon (Gausen & Moen 1991, Lura & Sægrov 1991), leading to genetic introgression (Glover et al. 2013, Karlsson et al. 2016) and resulting in changes in life-history traits (Bolstad et al. 2017, 2021) and phenology (Skaala et al. 2019) of wild Atlantic salmon. Whole-river controlled experiments in Ireland (McGinnity et al. 1997, 2003) and Norway (Fleming et al. 2000, Skaala et al. 2012, 2019) have shown that intrusion of farmed Atlantic salmon may result in a reduction of local population productivity and viability. This comes in addition to other challenges to wild Atlantic salmon caused by aquaculture, such as increased pressure from salmon lice and other disease agents, and other anthropogenic factors, such as hydropower regulation, introduced species, pollution, and climate change (VRL 2023).

In the mid-1980s, it was demonstrated that induction of triploidy in salmonid fishes, either by temperature or pressure shock shortly after fertilization, resulted in sterile individuals (Benfey & Sutterlin 1984). The method was inexpensive, technologically simple and could be applied with high yield and low mortality to large volumes of eggs. It was soon after used in salmon farming in Tasmania and Canada (Benfey 2001), and on rainbow trout (*Oncorhynchus mykiss*) released into ponds and reservoirs in Europe.

Norwegian aquaculture of Atlantic salmon has seen a tremendous growth since its inception in the late 1960s. The growth in production has been supported by a selection program for farmed Atlantic salmon from the early 1970s (Gjedrem & Baranski 2009), and by the development of feed, vaccines, and aquaculture technology. Increased production in open sea-cages has also been accompanied by increased problems related to escaped farmed salmon, infectious diseases, and pollution by chemicals, excrements and feed remains.

In 2009, the Norwegian government developed a strategy for sustainable aquaculture that listed a set of goals for the aquaculture industry, including limits to genetic and disease effects on wild Atlantic salmon. A renewed interest in basing aquaculture production on triploid salmon led to so-called “Research concessions” given to the study of triploid Atlantic salmon on an industrial scale and to “Green concessions” awarded to environmentally friendlier production, of which production based on triploid salmon were among the winning bids.

The results of this increased interest in triploid salmon led to experiments solving some of the bottlenecks to triploid production (Anon. 2015), but also raised welfare concerns based on large-scale studies of triploids in conventional aquaculture (Stien et al. 2023). Moreover, triploid salmon display reduced migration to freshwater (Glover et al. 2016). Male triploids develop gonads and secondary sexual characters and may take part in spawning, although not fertilizing eggs (Fjelldal et al. 2014). Therefore, as stated in the current application, the objective of the research trials is to develop genetically sterile diploid salmon as an alternative to triploid salmon.

The method employed to produce the genetically sterile fish for the research trials is based on CRISPR technology. CRISPR-Cas9 (clustered regularly interspaced palindromic repeats, and their associated (Cas) nucleases) was shown in 2012 to change DNA in a specific way, whereby small DNA changes, such as deletions and insertions (indels) of one to a few nucleotides could be targeted to specific DNA sequences of the genome (Doudna & Charpentier 2014, see also VKM Report 2021:18). This was a major methodological step forward in genetic modification of plants and animals, as previous genetic modifications were largely based on the insertion of novel DNA in random locations of the genome in these species. For example, the genetically modified AquaBounty salmon is based on inserting a transgene construct consisting of growth hormone from chinook salmon (*Oncorhynchus tshawytscha*), and an ocean pout, *Zoarces americanus*, antifreeze protein gene promoter into the genome of Atlantic salmon (Du et al. 1992). Selection among the offspring was used to demonstrate that this transgene was inherited in a stable way across generations (Yaskowiak et al. 2006).

The IMR-research group behind the current application has been in the forefront of research developing the CRISPR technique for Atlantic salmon and fish in general. In 2014, they showed that CRISPR-Cas9 could be used to knock out two pigmentation genes in the F0 generation, i.e., in the generation that is inserted with the CRISPR apparatus in the egg shortly after fertilization (Edvardsen et al. 2014). In 2016, they showed that germ cell-free (and thereby, sterile) salmon in the F0 generation can be produced by using CRISPR-Cas9 to knock out the expression of dead end *(dnd),* which is required for germ cell survival in vertebrates (Wargelius et al. 2016). In the same study, by generation of double knock out, they used albinism as a visual tracer to avoid studying mosaic individuals, since they found that induced mutations in the *alb* and *dnd* genes were highly correlated (Wargelius et al. 2016).

In 2022, they published a whole-generation study comparing F0 germ-cell free salmon with conventional farmed salmon in common garden tanks at the Matre Aquaculture Research Station (Kleppe et al. 2022). They found no difference in terms of deformities, smoltification, and fillet quality between F0 germ-cell free salmon and conventional farmed salmon but noticed a limited reduction in growth rate towards harvest size, which may be related to the conventional farmed salmon initiating puberty (Kleppe et al. 2022). The current release experiment into sea cages is meant as a prolongation of this study in a more realistic environment for industrial aquaculture production in sea cages.

The application includes fish generated by a new method for producing genetically sterile salmon, published by the same research group (Güralp et al. 2020). The research group has developed a rescue approach for producing germ cells in Atlantic salmon *dnd* knockouts *(dnd*-KO). To achieve this, they co-injected the wild-type (WT) variant of salmon *dnd* mRNA together with CRISPR-Cas9 constructs targeting *dnd* into 1-cell stage embryos (Güralp et al. 2020). As a result of this procedure, they are able to obtain F0 VIRGIN rescued individuals that contained germ cells. These F0 VIRGIN rescued individuals can be used to produce F1 germ-cell free salmon offspring, in a breeding where genetically sterile F0 salmon pass their sterility trait (homozygote for mutated *dnd*) on to the F1 generation (Güralp et al. 2020).

The research trial in this application is a result of a cross of such F0 rescued individuals made in November 2021. Hereafter, we call these offspring 'F1 VIRGIN' salmon. The F0 generation where the *dnd* gene is knocked out is called 'F0 VIRGIN' salmon (as studied in Kleppe et al. 2022) and 'F0 VIRGIN rescued' salmon if made physiologically fertile by *dnd* mRNA injection to compensate for the knocked out *dnd* gene (as in Güralp et al. 2020).

The mosaic nature of F0 VIRGIN salmon (e.g., Edvardsen et al. 2014, Wargelius et al. 2016) may result in a variety of germ cells that have incorporated one or two *dnd* knockout alleles (*dnd*-KO). Other cells may not have the same mutation(s) and therefore have wild type (WT) *dnd* alleles. Thus, from the same cross of two mosaic F0 VIRGIN rescued salmon, it may be possible to generate F1 embryos

that are *dnd*-KO/*dnd*-KO homozygotes and other F1 embryos that are WT/WT homozygotes. This approach has been used in the production of the experimental salmon included in the research trial and gives a rationale to explain how wildtype controls may be siblings of the F1 VIRGIN salmon.

The F1 VIRGIN material that was produced for the project is based on crosses of two F0 VIRGIN rescued males and four F0 VIRGIN rescued females. From each of the eight family groups from the two by four crosses, 300 offspring were kept in a pool of fish to be genotyped before inclusion of individuals to the study (altogether 2400 individuals). Genotype analyses of fin-clips of the adipose fin from 2200 individuals at one year of age gave 303 individuals that were homozygote *dnd*-KO/*dnd*-KO and 485 siblings that were WT/WT individuals. These siblings are intended as controls when studying welfare, behaviour and growth in sea cages.

The experiments will be carried out by releasing the 788 individuals at 1.5 years of age and average weight of 400 g in sea cages (open net pens) at the IMR Matre Aquaculture Research Station in September 2023. All individuals are PIT-tagged, vaccinated and length- and weight-measured before transfer to sea cages. The seawater rearing is carried out in double net pens where four 5 x 5 m net pens (5 m deep) are situated within a single 12 x 12 m net pen (14 m deep) at the Smørdalen Marine Facility (locality no. 12154 in the Norwegian Aquaculture register). This installation will be covered by double netting. Each of the 5 x 5 m nets will receive 197 (788/4) individuals that are monitored by PIT antennas at three depths, and sampled after 5 months, 10 months and at slaughter in February 2025 to investigate body size and welfare indicators such as malformations, injuries, and other potential health issues. Dead fish will be collected daily and environmental conditions recorded by CTD (conductivity, temperature, depth) profiling. Mesh sizes of the net pens follow Fisheries Directorate recommendations. If there is an escape, the applicant will follow standard procedures for capturing escaped farmed salmon.

The Smørdalen facility has access control, trained personnel, and the cages will be marked with GMO labeling. Fish that are euthanized or dead will be frozen in labeled plastic bags and maintained frozen until disposal through incineration. Upon detection of disease, the experiment will be terminated. If all fish survive until slaughter, the applicant expects approximately 1515 kg of experimental fish to be incinerated (303 individuals at 5 kg).

VKM assessed whether the information provided by the applicant is sufficient or whether there was a need for more information to perform a risk assessment of the GMO according to the Gene Technology Act § 15. The VKM risk assessment included assessment of risks to the environment and to animal health that can arise from the release trials. VKM also assessed the risk-reducing measures suggested in the application.

Details of the request to VKM are provided in the Terms of Reference.

1 Molecular characterization

Molecular characterisation is necessary to provide an insight on the genetic material introduced into the genetically modified animal genome and lays ground for the subsequent parts of the safety assessment as outlined in the Guidance on the risk assessment of food and feed from genetically modified animals and on animal health and welfare aspects (EFSA 2012). The molecular characterisation provides information on e.g., transformation method, the structure and expression of insert(s), deletion or modification, and description and stability of new trait(s). In addition, relevant information on the recipient organism, such as the species, subspecies, and breeding line, whether it is a common food staple, has a history of safe use, or whether information exists on known toxic or allergenic properties should be provided.

The main scientific documentation used in the VKM risk-assessment of VIRGIN salmon was the documentation and referenced publications provided in the application.

1.1 Information relating to the genetic modification

1.1.1 The Dnd gene

The *dead-end* gene (*dnd* in salmon) encodes *dnd* RNA-mediated repression inhibitor 1. The gene is localized to chromosome 5 (ssa 05-NC-059446.1). The transcript is according to publicly available databases spliced from 5 exons of either 1326 nucleotides (NM_001279131.1) or 1641 nucleotides (XM_014199425.2), giving rise to proteins with 360 (alternatively 366) or 367 amino acids, respectively, differing only at the N-terminus. Reference sequences are available here: https://www.ncbi.nlm.nih.gov/gene/101448053/

The dnd gene is found in vertebrates from fish to mammals and encodes a dnd protein containing RNA binding domains (Gross-Thebing and Raz 2020). The dnd protein can bind to specific sites in target mRNAs, and such binding prevents microRNAs from binding to the same 3'-untranslated region (UTR) of mRNAs where it would inhibit protein translation and sometimes also induce mRNA degradation. The dnd protein has several RNA binding domains and has been proposed to regulate protein translation in different ways in a variety of species depending on RNA and protein interaction partners, proposedly regulating differential protein expression in somatic and germ cells (Gross-Thebing & Raz 2020).

Dnd expression was first shown to be germ cell specific in zebrafish (Weidinger et al. 2003) and subsequently in other vertebrates, including several fish species. In fish, the expression of *dnd* is high already in the fertilized egg, thus of maternal origin. Its expression remains high until early gastrula but declines after the gastrula stage, and eventually *dnd* expression will only be detected in ovaries and testes. In *dnd* knock-down zebrafish, the germ cells are unable to migrate and reach the region where gonads normally would develop (Slanchev et al. 2009).

In VIRGIN salmon, expression of *dnd* has been knocked out with CRISPR-Cas9 technology resulting in individuals with double allelic loss of function mutations in the *dnd* gene in the F0 generation. Salmon with a double mutation in the *dnd* gene are found to be germ cell free (GCF), demonstrating that zygotic *dnd* transcription is required for fertility. Injections of the guide sequence (5'-

GGGCCCACGGCACGGAACAGCGG-3') and Cas9 mRNA was carried out after 3-4 hours of incubation of embryos at 6-8^{o} C after the fertilization of eggs with sperm. To produce offspring from sterile F0 salmon, mRNA encoding the dnd protein was injected together with the guide and Cas9 RNAs.

The accession number (JN71291127) given for Atlantic salmon *dnd* mRNA several places in the application is incorrect and gives no hit in the databases. We suspect that the last two digits should have been removed, as these seems to stem from a literature reference (27) in Wargelius et al. (2016) that have been incorporated in the accession number by mistake when copying text from that publication into the submitted application.

The (correct) accession number (Nagasawa et al. 2013) given on page 3 (JN712911.1) of the application gives a hit in GenBank that differs somewhat from the sequence shown on page 2 of the application, the difference being 13 versus 31 nucleotides prior to the first potential methionine start codon (ATG). While these sequences may be called mRNA sequences, they are given in DNA code, also called coding sequence (cds), which represents the sequence emerging after the introns have been removed. Since the above-mentioned nucleotide differences seem to be in the 5-end non-coding region, these discrepancies do not affect the protein coding region (cds).

The F0 generation can, as previously shown, obtain offspring (F1 VIRGIN) if co-injected with *dnd* mRNA at the embryonal stage. The used *dnd* mRNA was obtained by PCR amplification of ovarian cDNA from Atlantic salmon, followed by utilization of the generated PCR product as a template to generate a stabilized, 5'-capped and polyadenylated *dnd* mRNA, in one publication called "stabilized full-length wild-type *dnd* mRNA". These Atlantic salmon are called "rescued dnd crispants" (F0 VIRGIN rescued in this report) and may develop functional gonads containing germ cells (Güralp et al. 2020). Thus, the injected *dnd* mRNA has a longer half-life than the one that is maternally provided, sufficiently long to support the development of gonads and survival of germ cells (Güralp et al. 2020). For how long the injected *dnd* mRNA is detectable during development is not reported.

On page 3 of the application, a figure shows quantitation of the *dnd* mRNA in gonad tissue of wild type, germ cell free, and rescued male and female salmon. This figure is copied from Güralp et al. (2020), while the figure legend is missing. The RNA is isolated from gonadal tissue, also the residual gonad-like tissue of GCF salmon, where the latter displays no *dnd* expression. Wild type (WT) male and female salmon express similar levels of *dnd* mRNA in their gonads, relative to expression of the *ef1a* reference gene. These expression levels are quite different from what is shown in Wargelius et al. (2016), where the expression level in wild type male gonads is around 10% of that shown for females. This difference is striking but is not discussed in the application. It is difficult to know whether the difference is linked to the time of harvest of the fish, which was not precisely indicated, or is due to methodological differences or technical difficulties. The somewhat reduced (but still significant) levels of *dnd* mRNA quantified for rescued males and females must represent expression of mutated *dnd* mRNA transcribed from the mutated *dnd* allele. Translation of this mutated *dnd* mRNA is expected to lead to a dysfunctional protein, if any translation takes place. The expression of the dnd protein itself has not been quantified for Atlantic salmon, neither wild type, nor F0 VIRGIN (*dnd*-KO) salmon. The primers used to quantify the gonadal mRNA are found in the supplementary material of Wargelius et al. (2016):

Forward 5'-TCTGTACAGGGCCTGATGGT-3' and

Reverse 5’-TAAAACAAAGTAGGGGATCTGTG-3’

The first 5’ base of the forward primer anneals to nucleotide 1117 in the cds (JN712911.1) in GenBank, while the reverse primer is complementary to the nucleotides 1143-1166. Inserts and deletions generated by CRISPR are not expected to alter nucleotide sequences outside the target region. However, generation of a frameshift or introduction of a stop codon will change the genetic triplet code or generate premature protein termination, respectively. Therefore, expression and quantification of normal and mutated *dnd* transcription are expected to be similar, unless the gene editing produces a less stable mRNA.

The CRISPR targeting site in the *dnd* gene is localized in exon 3 at the codon for amino acid 72 (Güralp et al. 2020), which corresponds to the codon for amino acid 25 in the 78 amino acid long RNA binding domain (termed RNA recognition domain) in the dnd protein (Güralp et al. 2020). A description of the exon-intron structure of *dnd* and the domains of the expressed unmodified protein and their functions was not included in the original application but was provided in a later revision. The information helps to substantiate that the indels in question abolish the biological activity in the *dnd* knockouts.

The CRISPR sites are underlined for the two genes *alb* and *dnd* in Supplementary figure 1 in Wargelius et al. (2016). A note of caution is that there is a mistake in this figure, the *dnd* and *alb* sequences seem to have been interchanged for male knock out fish, when comparing to wild type and female knock out fish. The corresponding, complementary sequence of mRNA (shown as cds; TTCCGTGCCGTGGGCCC) derived from this target sequence corresponds to the bases 248-264 in the GenBank sequence (JN712911.1). This encodes the amino acids 71-78 (PLFRAVGP).

Alterations in the *dnd* gene of the fish included in the present application can only be analysed in tissue that can be removed non-invasively, preferentially by fin-clipping. Thus, the correlation between fin-clip samples, gonad formation, and function and fertility must be addressed by looking at previous work. For the fish included in this application, this can only be addressed retrospectively. In a previous study (Güralp et al. 2020) only 15% of the potentially rescued crispant fish had mutations in the *dnd* gene in fin clip samples analysed, while 1 of 5 (male) and 2 of 4 (female) examined did not develop into fertile animals. While animals without altered DNA based on fin clip analysis will be removed from the pool of fish with altered *dnd* gene, a more severe problem occur if *dnd*-KO fish have gonads with resemblance to wild type gonads. This was reported for 15 of 103 fish in Kleppe et al. (2022). Two of the *dnd*-KO individuals had germ cells due to a “lower mutation success”. Thus, while it is of importance that such individuals are identified by fin clip sampling, genotyping based solely on fin clipping is not a reliable method to determine the genotype in all tissues in founders (F0) that are genetically modified with CRISPR. If the natural allele (WT-allele) is modified at different cellular stages during development (typically at the 1-cell, 2-cell, or 4-cell stage), mosaic cells with different genomes may be formed. While each individual cell in the founder will have only two alleles, the founder (the F0 individual) may consist of mosaic cells with a higher number of allelic variants of the *dnd* gene. The presence of 3-6 allelic variants is not uncommon in founders generated with CRISPR. These cells with alternative genetic modifications may segregate at different ratios when specialized cell types and organs are formed. Güralp et al. (2020) present two individuals with high mutation frequency determined by fin clip that still had germ cells after analysis of gonad tissue samples. While it can be several biological reasons for these results, one alternative

explanation may be that a higher percentage of embryonic cells containing a functional *dnd* gene were unevenly distributed to anatomical areas that give rise to gonads. Another possible contributing factor is positive selection. Since expression of wild type *dnd* alleles is necessary for germ cell formation, cells with wild type *dnd* alleles will be positively selected to survive and differentiate into germ cells. Regardless of the biological explanation, the results show that fin clip genotype analysis alone is not a fully reliable method to determine gonadal genotype and/or formation in F0 individuals.

1.1.2 Description of the methods used for the genetic modification

As stated in the application, the presumed genetically sterile salmon, F1 VIRGIN (n = 303), intended for research in aquaculture sea-cages were produced in November 2021, and are offspring (F1) of F0 VIRGIN rescued salmon. The F0 parents were mutated in both alleles of the dead end (*dnd*) gene by injection of CRISPR-Cas9 reagents 3-4 hours after fertilization but received also *dnd* mRNA at the same time to enable germ cell formation despite the absence of the *dnd* gene (rescue) (Güralp et al. 2020). According to the applicant, the F1 VIRGIN salmon have inherited mutations in the *dnd* gene from both parents and lack germ cells.

In November 2021, eggs from four rescued females (F0 VIRGIN rescued) were fertilized with sperm from two rescued males (F0 VIRGIN rescued) (Table 1). Each male was crossed out to all females. About 300 juveniles from each cross were mixed at start feeding to create a group of about 2400 juveniles. At one year of age, 2200 salmon were pit tagged (November 2022) and genotyped. Leftover salmon were terminated. Of the 2200 salmon, 303 (F1 VIRGIN) had double allelic mutations in the *dnd* gene (13 % success rate). Production of the parent F0 VIRGIN rescued salmon is described below.

***Table 1**. An overview of mutation types in F0 parents used to create F1 VIRGIN salmon (n = 303).*

F0 VIRGIN rescued female	% Mutation in fin	Common mutations in *dnd* from mi-seq analysis	F0 VIRGIN rescued - male	% Mutation in fin	Common mutations in *dnd* from mi-seq analysis
402050	100%	Ins1/wt/del8/del7	9A49F	89%	del8/del7
620C6	100%	Ins1/wt/del8/del7	61E9D	97%	del8/del5
6228F	98%	del8/del7			
69762	98%	del8/del7			

The *dnd* gene mutations in the F0-parents were created using a CRISPR-Cas9 system as described by Wargelius et al. (2016) and Güralp et al. (2020). The *Salmo salar dnd* mRNA sequence (acc. JN712911) was used to find the genome scaffold containing the *dnd* gene (jcf1000428558_0-0, assembly Ssa_ASM_3.6. fasta (Acc. No. AGKD00000000.3)). The *dnd* target site was selected in the exon encoding the DNA-binding domain. To avoid off-target effects they searched the whole salmon genome (No. AGKD00000000.4) for binding sites for the nucleotide sequence selected for the CRISPR target for *dnd* (*dnd* guide RNA sequence: 5'-GGGCCCACGGCACGGAACAGCGG-3'). No unspecific binding was identified for the target sequence used. Cloning of CRISPR target sequence and

preparation of cas9 mRNA and *in vitro* transcription was conducted as previously described (Edvardsen et al. 2014), with some differences. In brief, *in vitro* transcription was conducted with the HiScribe T7 High Yield RNA Synthesis Kit (NEB) according to the manufacturer's protocol and template DNA was eliminated by incubation with 1 µl TURBO DNase (Ambion) at 37 °C for 15 min. The gRNA was purified using RNeasy column (Qiagen) according to the manufacturer's instructions. Cas9 mRNA was prepared by *in vitro* transcription as described previously for zebrafish (Jao et al. 2013). The QIAprep Spin Miniprep Kit (Qiagen) was used for the isolation, the mMessage mMachine T3 kit (Ambion) was used for *in vitro* transcription, and RNeasy column (Qiagen) was used for purification according to the manufacturers' instructions.

Full-length *dnd* mRNA was generated by PCR amplification of ovarian cDNA from Atlantic salmon using Q5 High-Fidelity DNA polymerase (NEB) and a forward primer that included a T7 promoter 5'-GAT TTA ATA CGA CTC ACT ATA GGG AAC AGA CCA CCA TGG AGG AGC GTT CAA GTC AGC AGG -3' with a reverse primer 5'-TTT GAC AAA TCT CAT TTT ATT ATA ATG AGA AAC AA-3'. The PCR product was extracted from a 1 % agarose gel, purified with QIAquick Gel Extraction Kit (Qiagen), and sequenced by Sanger sequencing. The *dnd* PCR product was used as a template to *in vitro* transcribe a functional 5'-capped and polyadenylated *dnd* mRNA using the HiScribe T7 ARCA mRNA Kit (NEB).

Preparation of embryos: Atlantic salmon eggs and sperm were obtained from AquaGen AS (Trondheim, Norway). The eggs were fertilized and treated with 0.5 mM L-glutathione reduced (Sigma-Aldrich) solution (pH 10) to prevent chorion hardening. Fertilized eggs were then incubated for 3–4 h at 6–8 °C to allow for blastodisc formation, at which stage the eggs were used for microinjection.

Experimental design and microinjections: The microinjection experiments took place at the Institute of Marine Research facilities at Matre Aquaculture Research station, Norway. gRNA targeting *dnd* and Cas9 mRNA with/without *dnd* mRNA was injected into a fertilized group of 1108 salmon embryos, as described above. Embryos were kept under hatchery conditions together with an untreated control group. At nine months post-fertilization, fin samples were collected from 60 fish and of five WT control fish to screen for mutations using High Resolution Melt (HRM) analysis followed by sequencing and qPCR analysis. At 11 months post-fertilization, gonad samples of the identified mutants were collected, and the remaining injected and untreated control fish were tagged, measured (weight and length), and fin clips were obtained for genotyping and subsequent sex determination. The most highly mutated broodstock (F0 parental groups; Table 1) were kept until autumn 2021 and crossed out to generate the F1 experimental and control groups. The applicant provided genotype data from fin clips of the 303 F1 VIRGIN salmon and their 485 WT sibling control salmon to be used in the applied research study (Appendix 1, Excel-file, overview of experimental fish).

Based on the genotypes presented for the parental fish in Table 1, it is difficult to interpret how the 485 double allelic wild type (WT/WT) siblings were produced, since only two of the parental F0 females and none of the parental F0 males have a WT-allele listed as a common variant.

For the F0-parental individuals, the CRISPR-Cas9 induced mutations in the *dnd* gene was screened by deep-sequencing, i.e., the part of the *dnd* gene containing the CRISPR-induced mutations. Genomic DNA from fin clips purified using Qiagen DNA extraction protocol was used as template for a two-step barcoding PCR targeting the CRISPR locus as described by Güralp et al. (2020). Briefly, each

sample was given a unique 6-mer barcode (Trueseq barcode primers) where after the barcoded *dnd* PCR products were mixed in equimolar ratios (oligos forward: 5' TCT TTC CCT ACA CGA CGC TCT TCC GAT CTC TAC ATG CAT CAT TC CCA CCC CA 3', reverse: 5' TGG AGT TCA GAC GTG TGC TCT TCC GAT CTA AG TTC CAC CAT TAC ACT GCT T 3'). The gene specific primers used span the intron–exon junction in exon3, where the CRISPR target site is placed. The final denatured sequencing library was prepared at a concentration of 8 pM and spiked with 5 % denatured phiX and sequenced on the Illumina MiSeq instrument using MiSeq Reagent Kit v3 (600 cycle format). No information on sequence depth is given in the application.

Mutation and variant analysis: The reads obtained from deep sequencing were filtered to only retain reads starting with the primer sequences (allowed 1 nucleotide mismatch in each primer sequence). The sequenced *dnd* amplicons obtained from forward and reverse template sequencing overlapped with 58 bp in their 3' ends, which allowed the reads to be merged if the overlap was at least 40 bp with at most 8 mismatches. For each overlapping base, the nucleotide with the highest base quality was used, yielding single forward reads covering the entire amplicon. These reads were cropped to only include positions 200–300 in each read, covering the *dnd* CRISPR site. Identical sequences were grouped, and unique sequences represented by only one single read were discarded to reduce noise from sequencing errors. The filtered sequences were aligned one-by-one to the amplicon reference sequence using Muscle (PMID: 15034147). Indels were identified and quantified for each sample (F0 individual) and were given names reflecting the 5' position of the indel in the amplicon sequence, and the size and type of indel (D = deletion, I = insertion). Reading frames for each indel combination was determined by the function 1 + [size (D)-size (I)] modulo 3, where size (D) and size (I) are the sizes of the identified deletions and insertions, respectively, in each sequence. The information about reading frames was used to calculate the proportions of in-frame and frameshift mutations in each sample. Indel variants represented at least 1% of the total reads in a sample.

Relative gene expression analysis: The applicant has provided a *dnd* gene expression analysis figure from Güralp et al. (2020, figure 3c and 3d). The *dnd* gene expression result represents both VIRGIN (germ-cell free salmon) and rescued VIRGIN salmon. The relative gene expression analysis method is as follows: total RNA was isolated from the gonad samples using a RNeasy Mini Kit (Qiagen) and DNase treated by TURBO DNase (Ambion), according to the manufacturers' instructions. The RNA concentration was determined by NanoDrop NP-1000 spectrophotometer (NanoDrop technologies, Wilmington, DE, USA). cDNA was synthesized with SuperScript VILO cDNA Synthesis Kit (Invitrogen) by following the protocol of the manufacturer. RT-qPCR reactions were set up for four replicates for *dnd* and run with *ef1a* as a reference gene), using PowerUp SYBR Green Master Mix (Applied Biosystems) according to the protocol of the manufacturer. The following *dnd* and *ef1a* gene primers were used: *dnd* gene (forward primer: 5'-TCTGTACAGGGCCTGATGGT-3' and reverse primer: 5'-TAAAACAAAGTAGGGGATCTGTG-3') and *ef1a* gene (forward primer: 5'-CCCCTCCAGGACGTTTACAAA-3' and reverse primer: 5'- CAGACGGCCCACAGGTACA-3'). The relative gene expressions were calculated by applying the method of Comparative Ct (or $2^{-\Delta\Delta Ct}$) (Applied Biosystems). The data were calibrated to the sample with the highest gene expression.

The application provided sufficient information about methods used to produce VIRGIN salmon and F0 VIRGIN rescued salmon, but very limited data for identification of the 303 F1 VIRGIN salmon and the 485 WT sibling control salmon to be used in the study. E.g., a detailed description of the method used for genotyping should be included in the application. The applicant provided limited information regarding the F1 VIRGIN salmon, n=7 *dnd*-KO F1 VIRGIN and n=9 WT fish from the 2021 season sampled in September 2022. These 7 *dnd*-KO F1 VIRGIN salmon (same crossing) were siblings of the 303 F1 VIRGIN salmon intended for release into sea cages. One gonad for histological analysis, one gonad for gene expression analysis, and fin clip to determine the *dnd* mutation type were used

from each salmon. The gross morphology (7 *dnd*-KO F1 VIRGIN and 2 WT salmon), gonad histology (4 of 7 *dnd*-KO F1 VIRGIN and 2 WT salmon), genotype (7 *dnd*-KO F1 VIRGIN and 9 WT salmon), and gene expression of *dnd* and *vasa* genes (7 *dnd*-KO F1 VIRGIN and 9 WT salmon) from gonads of F1 VIRGIN fish were included in the application. Still, this information did not cover all *dnd* allelic variants present in the 303 F1 VIRGIN individuals.

There are also concerns regarding the genotyping results. F0 founders generated with CRISPR typically are mosaic, with multiple alleles for *dnd* gene. All individuals produced by crossing of such mosaic founders will, however, inherit only one of these multiple alleles from each parent. The two alleles inherited for each individual fish can be determined, but the reliability of their assessment depends on the methodology used. A low number of individuals may potentially be assigned to the wrong genotype when thousands of individuals are screened. There are several possible scenarios that may result in incorrect assessment of the genotype and/or inclusion of an individual with a wrong genotype using PCR genotyping: 1) inefficient DNA extraction for some samples and/or contamination may generate false negative or false positive signals. 2) sample mix-up when biopsies are taken/wrong marking of individuals, 3) a correctly genotyped fish may be mixed up with another individual. To verify that no such errors occur, a confirmative genotype analysis with a new sample from all individuals should be conducted, i.e., samples from the 303 F1 VIRGIN and the 485 WT salmon. Sampling must be performed after selecting the 303 F1 VIRGIN and the 485 WT salmon from the heterozygous siblings not intended to be included in the experiment. It is unclear whether such a re-confirmation of genotypes was performed.

1.2 Information relating to the GM animal

1.2.1 General description of the trait(s) and characteristics introduced or modified

According to the application, the *dnd* gene is only expressed in the germ cells. The F0 VIRGIN salmon is made sterile by parental CRISPR-double allelic mutations leading to a frameshift in the gene sequence and loss of function of the *dnd* gene responsible for germ cell maturation and migration. The applicant states that: "If there is an out-of-frame mutation, it results in the absence of a functional protein. In other words, it is a loss-of-function mutation. All 303 fish listed in the application have double allelic, loss-of-function mutations, which means they do not produce the dnd protein, and the cells that would normally produce this protein are also absent."

In a study by the applicant published in 2020, the authors present a linear correlation between the rate of frameshift mutation and the expression of *dnd*, showing a possibility of rescuing the function of the dnd protein by mRNA microinjection where only a few frameshift mutations were present (Güralp et al. 2020).

The F1 VIRGIN salmon has according to the applicant loss of function mutations in the *dnd* gene, without vector or CRISPR-sequences inserted in the genome. The applicant states that DNA from fin clip of all fish were sequenced without providing the sequencing data in the application.

The applicant analysed 28 *dnd*-KO (loss-of-function) salmon from an earlier crossing of F0 VIRGIN rescued salmon in 2020 (unpublished data, included in the application), sectioned the gonads, and found that they all lacked germ cells, and lacked expression of the germ cell marker *vasa* (referred in the application) without providing the vasa gene expression data in the application. Based on the assumption that all 303 F1 VIRGIN salmon have proven double allelic loss-of-function mutations, they are all expected to be sterile.

According to the applicant, the germ cell free (GCF) CRISPR-Cas9 salmon from previous studies in indoor tank facilities were observed to not undergo puberty (Kleppe et al. 2017). The applicant has followed GCF salmon through a complete life cycle in indoor facilities and found that they cannot be

distinguished from control fish in terms of deformities, smoltification, fillet quality, and growth (Kleppe et al. 2022). According to earlier studies on germ cell free salmon, the immature WT group became heavier than GCF females (Kleppe et al. 2017) due to higher initial weights at start of the experiment, but without significant differences in growth rates. The authors state that there are no negative effects on growth in GCF salmon females. Time to full maturity was, however, not followed in that study. Sex hormone levels in GCF female and male salmon were very low or undetectable in the same study (*dnd*-KO = 28, WT = 45). Further, they showed that GCF testes failed to produce a signal required to enable the brain-pituitary system to respond to the stimulatory photoperiod/ temperature cues, keeping the fish in a prepubertal state. None of the 28 GCF female nor male salmon showed any sign of maturation in that study (Kleppe et al. 2017). The 28 GCF in the study by Kleppe (2017) were produced as described in the previous study by Wargelius (2016) via CRISPR-Cas9 and no information on the type of *dnd* mutations were provided. In the study by Kleppe et al. (2022), most welfare markers and body size displayed similar values in the GCF (also directly via CRISPR-Cas9) and WT groups, while the relative content of EPA and DHA were higher in GCF compared to WT male despite the same amount of total omega-3 fatty acids (Kleppe et al. 2022,). Again, the authors showed that the GCF salmon remained immature, up to 1.5 years of age and with lower or even undetectable plasma sex steroid levels compared to WT (estradiol-17β (E2), 11-ketotestosterone (11-KT) and testosterone), while the rate of WT maturation at harvest was 10% (Kleppe et al. 2022.). GCF fish could not be distinguished from WT regarding body size, smoltification markers, plasma stress indicators (pH, glucose, sodium, chloride, calcium), relative heart size, prevalence of vertebra deformities, and fillet proximate composition. Transient differences were detected in plasma concentrations of lactate and osmolality, and only a few genes were differentially expressed in WT- and GCF transcriptomes of muscle and pituitary. Kleppe et al. (2022) found a higher growth rate towards harvest size, as well as higher condition factor and relative liver size in WT than in GCF fish, probably relating to initiation of puberty in WTs (Kleppe et al. 2022.). The study included 94 *dnd*-KO GCF salmon and 148 WT fish. The overall mortality was similar for the WT and *dnd*-KO fish (12.5 and 8.9%, respectively). At the start of the experiment, 15 of 103 gross-morphologically analysed fish were detected to have WT-like gonads, confirmed with normal expression of *vasa* and 100% WT *dnd* sequence (omitted from further analysis). No *dnd* sequences were provided, but the % WT *dnd* indel sequence (homology) was between 0.00-0.15 (similar calculation in two WT fish gave 99.36 and 99.37% WT sequence). The *dnd*-KO fish with normal gonad gross morphology and presence of germ cells had 26.72 and 66.56% WT *dnd* sequence. The WT *dnd* sequence percentage in fin compared to gonad tissues in these two *dnd*-KO fish was 17 versus 27 and 31 versus 67%, respectively (Kleppe et al. 2022). This indicates some variability when extrapolating genotype from fin clip to gonad. As a visual tracer in these experiments, the *dnd*-KO fish also had an introduced mutation in the *slc45a2* gene leading to loss of pigmentation.

The applicant states that no diseases have been observed in the experiments performed in indoor tanks in the fish that were unvaccinated. The health status of the VIRGIN salmon in the indoor containment to be used in the applied research study is based on the veterinary reports from Matre. The reports state information on group mortality, however, no individual health status is available. Additional references on germ cell free albino fish indicate a health similar to that of the wild type in indoor facilities (Kleppe et al. 2022). While sterility is the intended outcome, sterility in itself can have significant implications for the produced individuals. Regarding characteristics and nutritional constituents, they have been shown to remain within similar range as the control fish until they reach maturity, especially for the most common *dnd* mutations (del8 or del7) in germ cell free fish.

1.2.2 Information on the sequences actually inserted/deleted or altered

Intended modifications and actual modifications will very often differ at some level. Information should therefore be available describing all detectable modifications, including unintended insertions, deletions and alterations and their positioning in the genome. There should also be information on whether insertions or deletions have unintentionally interrupted the regulation/expression of existing genes in the organism. All new open reading frames (ORFs) should also be investigated in and around all sites of modification. New ORFs are sequences that may potentially lead to the expression of unwanted harmful products such as allergens or toxins.

According to the application, no foreign DNA sequence(s) has been inserted into the genome in the F1 VIRGIN Salmon. The *dnd* gene was mutated using the protocol stated above. The most common mutations found in *dnd* are listed in Güralp et al. (2020). The most common mutations that lead to loss-of function in the F0 parents were del8/del7/del5 and Ins1. The most common variant among all mutants was an 8 bp deletion [236-8D (3), TTCCGCTG] causing a shift in the reading frame generating a PTC (premature termination codons) at amino acid (aa) 159 in exon 4. The second most common variant was a 7 bp deletion [242-7D (2), TGTTCCG] generating an early PTCs at aa 86–87 in exon 3. Of the fish previously characterized as rescued, 70% have some degree of WT *dnd* in their genomes, similar to 33% of the GCF fish. The different *dnd* mutation variants of the 303 F1 VIRGIN salmon are presented in Appendix 1 (Appendix 1, overview of experimental fish provided by the applicant).

In Güralp et al. (2020), the indels affecting the reading frame were rated 1-3, where reading frame (1) does not affect the overall amino acid sequence, while reading frames (2) and (3) represent frameshifts with distorted amino acid sequence downstream of the CRISPR site (Table 2). Frameshift mutations in reading frames (2) and (3) caused incorrect coding-reading after the modification site and introduction of premature termination codons (PTCs) at different locations in the mutated mRNA.

Table 2. Unique indel variants in germ cell free gonads and positions of the PTCs (represented by at least 1% of the total reads per sample) from Güralp et al. (2020).

Sample ID	Indel	Reading frame	Reads in gonad		Position of PTC	
			quantity	rate	exon	aa
18	244-11I	3	13897	38.39%	3	86-87
15	240-16D	2	5582	14.7%	3	86-87
15	231-17D	3	4906	12.92%	4	159
16	240-73D	2	4818	14.27%	4	159
20	219-33D	1	4050	13.66%	-	-
21	240-1D	2	3646	12.81%	3	86-87

Note. I: insertion, D: deletion, aa: amino acid, PTC: premature termination codon. Supplementary Table S4 in Güralp et al. 2020.

In a later revision of the application (September 2023), the applicant provided updated genotype data of the F1 VIRGIN salmon (n=303) in a new Excel-file. The file contained information on frameshift mutations including nucleotide location (number 5´-3´) of the indel(s) and a table in the application with reading frame information and amino acid position of premature stop codon.

According to the application and Güralp et al. (2020), the most common mutation types in the F0 VIRGIN rescued salmons leading to loss-of function mutations are del8/del7/del5 and Ins1. In Table 3, the distribution of *dnd* mutation types (genotype data from Appendix 1; Excel-file; overview of experimental fish provided by the applicant) for the 303 F1 VIRGIN salmon are presented. Around 7% of the 303 F1 VIRGIN salmon (21 out of the 303 F1 VIRGIN salmon) had less common *dnd* mutation types (Table 3). The different mutation variants of the 303 F1 VIRGIN salmon with common name of the indels and the position of the premature termination codon are presented in Table 4.

Phenotypic information on salmon with these less common *dnd* mutation types, i.e., gross morphological, histological or gene expression (*dnd* or *vasa*) data were not provided in the application. It is not sufficient with genotype data to assess the 7% F1 VIRGIN salmon with less common *dnd* mutation types. Therefore, a more detailed information about these less common *dnd* mutation types would help the risk assessment of the 303 F1 VIRGIN salmon.

Table 3: Distribution of the 303 F1 VIRGIN salmon *dnd* mutation types (Appendix 1, Excel-file)

***dnd* mutation type**[(*)]	**Number of *dnd* mutation variant**	**Frequency (%)**
del8/del8	138	45.5
del8/del7	31	10.2
del7/del5	28	9.2
del8/del10	22	7.3
del8/del5	19	6.3
del10/del10	13	4.3
del10/del5	9	3.0
del8/ins1	8	2.6
del8/del2	7	2.3
del7/del10	5	1.7
Ins1/Ins1	3	1.0
del2/del2	2	0.7
del8/del4	2	0.7
del8/Ins10	2	0.7
del8/Ins5	2	0.7
Ins5/Ins10	2	0.7
del2/Ins5	1	0.3
del28/del28	1	0.3
del28/Ins5	1	0.3

del4/del4	1	0.3
del7/del2	1	0.3
del7/del28	1	0.3
del8/del2	1	0.3
del8/ins4	1	0.3
Ins1/del28	1	0.3
Ins1/ins5	1	0.3

*Note. * Less common dnd mutation types are colored in red. According to Güralp et al. (2020) and the application, the most* common loss of function *variants in the dnd gene were ins1, del8 and del7.*

The data provided in a late revision (version 4. September 2023) of the application included genotype data from 7 F1 VIRGIN salmon (from season 2021) and 28 *dnd*-KO F1 fish (from season 2020, old crossing). The majority of these fish have the most common mutation types (del8/del7/del5 and Ins1, all loss-of function mutations). All the 7 F1 VIRGIN salmon have Ins1/del8 mutation types, while 93 % of the 28 *dnd*-KO F1 fish have del8/del8 and del7/del7 mutation types. Only one female and one male fish among the 28 *dnd*-KO F1 fish have less common mutation types (Ins2/Ins2). The new information provided in September did not cover the less common mutation types observed in the 303 F1 VIRGIN salmon. These 28 *dnd*-KO fish are offspring of a different batch of F0 VIRGIN rescued fish from season 2020 (old crossing).

According to the application, all 28 *dnd*-KO fish lacked germ cells (based on gonad histology) and all lacked expression of the germ cell marker *vasa*. The *dnd* genotype variants of the 28 dissected *dnd*-KO salmon were to some extent present in the 303 VIRGIN salmon in the application. These 28 *dnd*-KO fish are offspring of a different batch of F0 VIRGIN rescued fish from season 2020 (old crossing). However, only representative microscopic histological data of ovaries and testes from WT (n=2, one ovary and one testis) and F1 *dnd-KO* (n=2; one ovary and one testis) was included in the application and no *vasa* gene expression data was included. The limited data provided for the 28 *dnd*-KO and 7 F1 VIRGIN salmon are not sufficient to perform a risk assessment of all 303 F1 VIRGIN salmon.

ALLELE VARIANT	MUTATION TYPE	Reading frame	Aa Position of premature stop codon	Site where erroneous Protein code starts
226-8D	DEL-8	2	aa155	aa75
224-7D	DEL-7	3	aa83	aa74
225-8D	DEL-8	3	aa155	aa75
220-10D	DEL-10	3	aa83	aa73
225-5D	DEL-5	3	aa83	aa75
231-1I	INS-1	3	aa83	aa77
232-8D	DEL-8	2	aa155	aa77
230-28D	DEL-28	2	aa155	aa77
229-2D	DEL-2	2	aa155	aa76
231-5I	INS-5	3	aa83	aa77
227-4D	DEL-4	2	aa155	aa75
223-10D	DEL-10	2	aa155	aa74
231-10I	INS-10	2	aa155	aa77
228-5D	DEL-5	3	aa83	aa76
229-28D	DEL-28	2	aa155	aa76

Table 4. Mutation variants among the 303 F1 VIRGIN salmon, common name of the indel, reading frame of the indel, premature termination codon and site where erroneous protein code starts (copied from the application).

1.2.3 Information on the expression of the inserted/modified sequence

The applicant has provided a figure of *dnd* gene expression analysis obtained from Güralp et al. (2020, figure 3c and 3d). The relative gene expression levels (relative to the expression of the *ef1a* gene) were calculated by applying the method of Comparative Ct (or $2^{-\Delta\Delta Ct}$) (Applied Biosystems). The Ct-values (ΔCt-values) were normalised against the group with the highest gene expression levels and show *dnd* mRNA expression levels for WT salmon (WT males), VIRGIN salmon (germ-cell free males) and VIRGIN rescued salmon (Rescued males) in smolt-sized immature fish. Germ cell free fish (equivalent to F1 VIRGIN salmon) do not express the *dnd* gene either in males or females. The exact age of the fish when gonads were sampled is not stated in Güralp et al. (2020) but was between 11 and 16 months post fertilization. Fish were harvested from two different experiments, with or without additional knock out of *slc45a2*, which generates albino fish.

In September (2023), the applicant provided additional data on the relative gene expression levels of *vasa* and *dnd* in WT (n = 9) and *dnd*-KO (n = 7) ovaries and testes. This information was obtained from 10 months old fish using one of the sibling families included in the 303 fish applied to be put into sea. The expression level of *vasa* gene was used as a marker for fish lacking germ cells in the gonad, and therefore, confirming the sterility of the fish, while the *dnd* expression level to confirm the loss of function. No expression of *vasa* or *dnd* mRNA was observed in either female or male *dnd*-KO fish (see Figure 1 (figure 3c & 3d in Güralp et al. 2020)). The ratio of *dnd* mRNA expression in WT females and males appears to be similar (Figure 1) since expression levels were normalized to WT independently for each gender (Güralp et al. 2020). In the newer figure (September 2023) in the revised application (Figure 2), the expression in male gonads is approximately 3 times that in female gonads for 10 months old fish. In yet another analysis performed by the applicants (Wargelius et al. 2016; Supplementary figure 5), the expression of *dnd* mRNA is approximately 10 times higher in female salmon compared to male salmon. These fish were harvested 12-18 months post fertilization. The variability in the *dnd* mRNA expression levels between male and female fish is the only parameter possible to compare for the presented data, since the expression level is given in arbitrary numbers, relative to a reference gene. The variability in the expression level ratio of the *dnd* gene in male to female WT fish is either (1) a result of variability in expression levels at different times of harvest, (2) from experiment to experiment, or (3) a problem with the accuracy of the method.

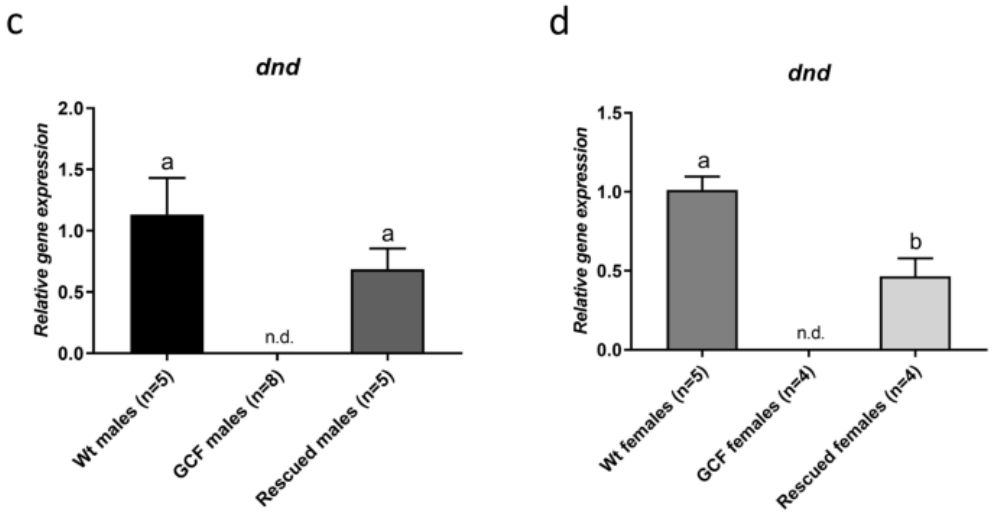

Figure 1: Gene expression in the gonads of *dnd* GCF fish and *dnd* fish with rescued germ cells compared to WT controls (figure 3 from Güralp et al. 2020).

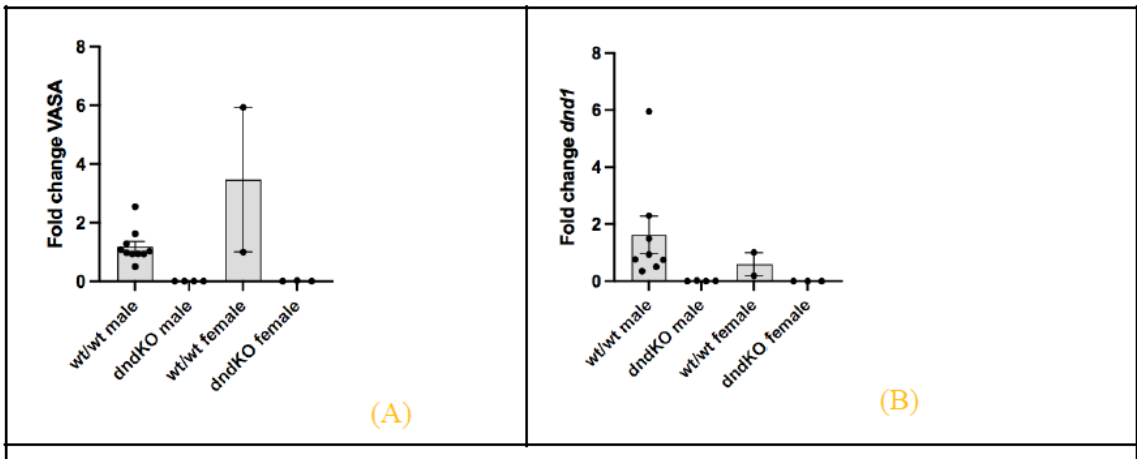

Figure 2. Relative gene expression of *vasa* (A) and *dnd* (B) in WT and *dnd*-KO ovaries and testes, obtained from 10 months old fish, using one of the sibling families included in the 303, fish applied to put into sea (new figure from applicant, September 2023).

According to the application, the selected *dnd* mutations are loss-of-function mutations, resulting in the absence of dnd protein expression. Since the cells that express the dnd protein (germ cells) also are missing, there is a lack of dnd protein in the F1 VIRGIN salmon. Further, the applicant does not have an antibody that recognizes the dnd protein. Very few antibodies have been made in fish and it would be expected that the process of making such fish antibodies would take 1-2 years in salmon. It might be possible to use mass spectrometry, although it is more expensive and labor-intensive. This method would confirm the absence of the dnd protein or the presence of truncated versions in the VIRGIN salmon. The dnd protein is exclusively expressed in germ cells, which are not present in VIRGIN salmon (Wargelius et al. 2016).

The application provided information demonstrating that the modified (edited) sequences resulted in intended changes at the gene expression level for the F0 VIRGIN rescued salmon (parents of the F1 VIRGIN salmon) and GCF (VIRGIN salmon) fish. However, it is important to note that the GCF fish are not offspring of the F0 VIRGIN rescued salmon, i.e., the GCF fish are produced by a direct CRISPR-Cas9 mediated modification. On the contrary, the 303 F1 VIRGIN salmon are produced by crossing rescued F0 VIRGIN male and female salmon, i.e., they inherited a loss-of-function mutation in the *dnd* gene from their parents (F0 VIRGIN rescued salmon). Therefore, the *dnd* gene expression level data provided in the application is not from individuals equivalent to the 303 F1 VIRGIN salmon, as stated in the application version 3.

In the latest revision of the application (September 2023), the applicant provided the relative gene expression levels of *vasa* and *dnd* in WT and *dnd*-KO ovaries and testes, obtained from 10 months old fish, using one of the sibling families included in the 303 F1 VIRGIN salmon applied to put into sea.

VKM concludes that the applicant has not conducted analyses of dnd protein levels in the 303 F1 VIRGIN salmon. The arguments provided in the application not to perform protein analysis are on the other hand satisfactory, since additional histological data of a few fish (n = 4 fish) equivalent to the 303 F1 VIRGIN salmon applied to put into sea now is included in the application. Alternative methods, such as *in situ* hybridization with anti-sense RNA, might provide supportive information when antibodies for specific protein detection are not available. First at the end of the planned experiment, the applicant will know whether the genotype data extracted from the fin clip correspond to the gonads and actual infertility in all *dnd*-KO individuals.

1.2.4 *Inheritance and genetic stability of the inserted/modified sequence and phenotypic stability of the GM animal*

In the application, it is stated that the F1 VIRGIN salmon contain double allelic loss-of-function mutations in the *dnd* gene, which means that they do not contain germ cells with dnd proteins. In the response (June 2023) to questions from VKM, it is stated that: "The genetic stability of the dnd mutation is similar to that of any other SNP (Single Nucleotide Polymorphism) or small genetic change found in the genome of any organism. Therefore, there is no reason to believe that this genetic variation would be any less stable than other genetic variants present in the genomes of living organisms." However, data of inheritance have only been provided for one generation in this application (F1 VIRGIN salmon generated from F0 VIRGIN rescued salmon).

VKM concludes that information about the inheritance patterns and the phenotypic stability of the introduced traits is lacking in the application. The data provided in the application are from F0 VIRGIN rescued salmon (F0-parents) and with very limited data for F1 VIRGIN salmon (fish equivalent to the 303 F1 VIRGIN salmon). In their last paragraph, Güralp et al. (2020) stated that *"In this regard, further studies will focus on examining the quality of rescued gametes, as well as investigating the germline transmission of dnd mutations, and will be followed by examinations of phenotypes and genotypes of juveniles in the F1 generation*." The authors acknowledge that there is a need for further investigations about phenotypes and genotypes of juveniles in the F1 generation. The application does not state whether such investigations have been conducted. Information about inheritance patterns and phenotypic stability of introduced traits are important parts of the molecular characterization of GM animals.

1.2.5 *Off-target effect*

Unintended changes resulting from genetic modification go beyond the planned changes resulting from genetic modification. The applicant has performed BLAST search of the guide RNA (gRNA sequence of *dnd*: 5'-GGGCCCACGGCACGGAACAGCGG-3') used to knock out the *dnd* gene (Wargelius, Leininger et al. 2016, Güralp, Skaftnesmo et al. 2020) in the F0 -generation (parents of F1 VIRGIN salmon) against the most recent salmon genome version (version Ssal_v3.1).

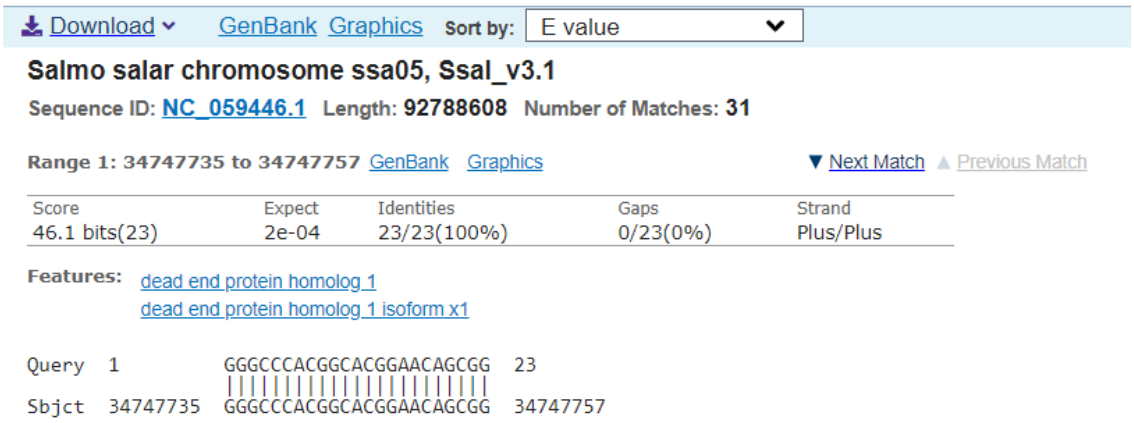

Figure 3: BLAST search result for gRNA sequence (best hit)

The guide RNA recognizes one location in the salmon genome (version Ssal_v3.1), i.e., in the *dnd* gene on chromosome 5 (Figure 3). There is no indication that unintended changes resulting from genetic modification could occur, since the guide RNA seems to have 100 % homology to only one area in the genome in (the *dnd* gene).

Further, the guide RNA sequence has a 95% match in the genome on chromosome 9, but this match lacks the last three nucleotides at the 3' end, which constitutes the PAM site (NGG) necessary for Cas9 to cleave in this region. Therefore, it is highly unlikely that it will induce a mutation on this site (Figure 4). The E-value was only good for the *dnd* gene, and the other matches in the salmon genome (version Ssal_v3.1) showed low similarity to the guide RNA sequence.

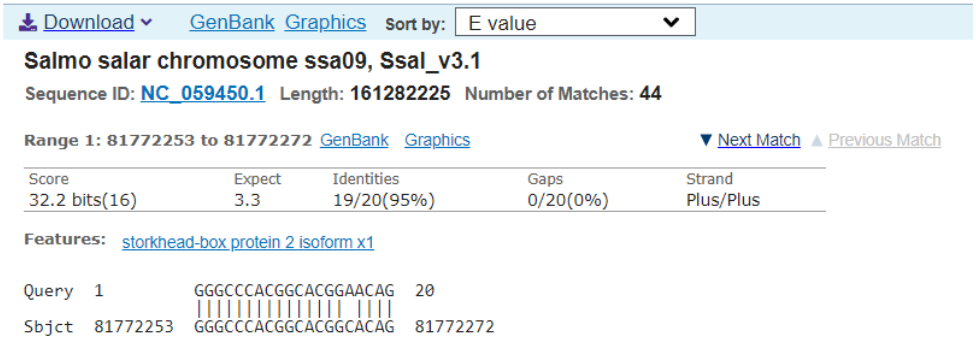

Figure 4: BLAST search result for gRNA sequence (second best hit)

The application provides a BLAST search result of the guide RNA sequence, and the search result and the arguments provided in the application are reasonable. There are no indications that the guide RNA used is likely to result in unspecific alterations of the salmon genome.

1.3 Conclusions of the molecular characterization

The applicant has provided literature references with sufficient information about the applied methods to produce germ cell free (GCF), i.e., sterile salmon, by direct CRISPR-Cas9 mediated double-allelic knockout (KO) of the *dnd* gene in fish embryos. Likewise, sufficient information is provided in the application for the methods used to develop the genetically sterile but functionally fertile salmon called "F0 VIRGIN rescued". The development of F0 VIRGIN rescued salmon includes micro-injections of *dnd* mRNA together with CRISPR reagents into 1-cell stage embryos, facilitating germ cell migration and gonad formation "rescuing" the fertility in the genetically sterile fish.

Two F0 VIRGIN rescued males and four F0 VIRGIN rescued females were used to produce purposely sterile F1 VIRGIN salmon intended for release into sea-cages.

A total of 2400 F1 VIRGIN salmon were produced from eight family groups with two males and four females each, out of which 2200 were genotyped by fin-clips of the adipose fin. The fin-clips identified 303 homozygous individuals that were mutated in both alleles of the *dnd* gene (mut/mut) and 485 homozygous individuals that had naturally occurring, i.e., normal wild type (W), versions in both alleles (WT/WT). The 485 (WT/WT) individuals/siblings are intended as controls (comparators) to the 303 (mut/mut) individuals when studying welfare, behavior, and growth in sea cages.

Limited data is provided in the application regarding the offspring (F1 VIRGIN salmon), e.g., the method used for genotype analysis is not sufficiently described. This information is relevant in the assessment of the accuracy of the genotyping. Also, a second genotyping should have been

performed to confirm the genotypes of the selected individuals (n = 788), to avoid unwanted inclusion of potential heterozygous individuals, such as those resulting from erroneous sampling or other possible confounders.

Moreover, the application contains insufficient data to confirm that all 303 F1 VIRGIN salmon are sterile, even if accurately genotyped. Double-allelic mutations in the *dnd* gene are indicated for all 303 individuals, but not all these mutations are confirmed to lead to a loss-of-function, even though this is theoretically likely the case. The application presents genotype data on 28 *dnd*-KO (loss-of-function) salmon that are also F1 offspring from F0 VIRGIN rescued salmon from a different batch. Around 93 % (26 out of 28 fish) of these 28 *dnd*-KO fish have del7/del7 (18 fish) or del8/del8 (8 fish) mutation types. These mutation types are also present among the 303 F1 VIRGIN salmon in question, providing some support to the assumption that those individuals out of the 303 F1 VIRGIN salmon are equally germ cell free. However, about 20 of the 303 individuals have other types of *dnd* mutations than those shown for the 28 *dnd*-KO individuals. Further, the application stated that these 28 *dnd*-KO fish were dissected and proven to lack germ cells and expression of the germ cell marker *vasa*. However, only representative microscopic histological data of ovaries and testes from WT (n=2, one ovary and one testis) and F1 *dnd*-KO (n=2; one ovary and one testis) were included in the application and no *vasa* gene expression data was provided in the application. The limited data provided in the application for the 28 *dnd*-KO fish are not sufficient to be used in the risk assessment of the 303 F1 VIRGIN salmon.

Histological data have only been provided for four fish from the same crossing as the 303 F1 VIRGIN salmon (one ovary and three testis). The gene expression data of the *dnd* and *vasa* genes from 7 F1 VIRGIN salmon and 9 WT salmon are presented in the application. These 7 F1 VIRGIN salmon have Ins1/del8 mutation types and did not represent all the mutation variants present in the 303 F1 VIRGIN salmon.

VKM concludes that the data provided in the application is not sufficient to confirm that the genotypes of all 303 homozygous (mut/mut) F1 VIRGIN salmon and all 485 homozygous (WT/WT) fish are correct for all individuals.

Furthermore, VKM concludes that it is not sufficiently documented that all genotypes identified among the 303 F1 VIRGIN salmon indeed result in germ cell free and/or sterile individuals. Some of the double allelic mutations identified among the 303 F1 VIRGIN salmon are assumed by the applicant to result in germ cell free sterile fish, but this assumption is not supported by confirmative experimental data in the application.

Overall, based on the information provided in the application, VKM concludes that there is a possibility that an unknown number of potentially fertile individuals of F1 VIRGIN salmon with allelic mutation(s) in the *dnd* gene are erroneously registered as double allelic mutants (n = 303). Likewise, an unknown number of the homozygous wildtype controls (n = 485) may in fact be fertile heterozygous fish, carrying a mutated *dnd* allele.

2 Cross-cutting considerations

These are fundamental considerations that permeate the individual parts of an environmental risk assessment and constitute key information that risk assessors require in order to perform a sound risk assessment.

2.1 Receiving environments

2.1.1 Definition of receiving environments

The receiving environment for the application is a set of experimental net pens at Matre Aquaculture Research Station in Norway. These net pens are a modified set-up of conventional net pens used in aquaculture, but at a mini scale in comparison with current conventional aquaculture.
Net-pen (or sea-cage) aquaculture are confined by nets on all sides and at the bottom so that sea water can flow freely through the production facility. This design makes the production facility resemble the environment of free-swimming fish, but also makes the production facility interact with the surrounding environment with respect to disease agents, food waste, and excrements. In addition, escapes of farmed fish are known to occur from net pens. The receiving environment must therefore include the surroundings of the experimental net pens that shall be used in the application. The geographical extent of the surrounding environment depends on the hazard that is studied.

2.1.2 Identification and characterization of the receiving environments

The experiments shall be carried out in open net pen aquaculture at the Smørdalen site (#12154 in the Fisheries Directorate aquaculture register) at the Matre Aquaculture Research Station in Nordhordland. The intended start of the experiment is September 2023 when the experimental fish shall be released into net pens at an average size of 400 g (estimated in late September 2023). This is in their second year as free-living individuals and in fish farming, they are commonly referred to as “large smolt” or “autumn smolt”. Wild Atlantic salmon at this size would be referred to as “post-smolt” (i.e., after smolt migration into sea water).

The experimental fish material used is 303 F1 VIRGIN salmon and 485 sibling WT salmon as controls. The net pens used in the experiment will not be standard net pens, but four smaller cages (5x5 m) tied and anchored within a 14 m deep larger net (12x12 m).

Each of the small cages is 5 m deep and thus has a volume of 125 m^3. In present-day aquaculture, a standard sea cage has a depth of 20-50 m and a diameter of 50 m. Thus, the volume of a typical sea cage is between 39 000 and 98 000 m^3. During the growth of the industry, the development of equipment in commercial fish farming has led to increasingly large sea cages (McIntosh et al. 2022).

The experimental fish will be fed by standard feed and held in the net pens for a period of 18 months. The extent of the period follows the common production pattern in commercial aquaculture from autumn release of smolt-sized individuals until slaughter. The expected density of salmon in the experiment has not been calculated by the applicant, but if fish are held until an average size of around 5 kg, then the density at the end of the production period is well within the limits set by the authorities at 25 kg per m^3 volume of the net pen (Anon. 2010).

In addition to the intended receiving environment, there is also an unintended receiving environment, because some of the fish may escape to the environment. Depending on time of year and life stage of the individuals that escape, the unintended receiving environment may range from

the local fjord and nearby rivers to the open ocean and rivers hundreds of kilometers away (Hansen 2006, Skilbrei et al. 2015). This is described in Chapter 3 of this report.

Site 12154 is located in Production area 4 (PO4 from Nordhordland to Stadt). Within PO4, both Atlantic salmon and rainbow trout are produced. PO4 was coloured red according to the Traffic light system (Fisheries Directorate), i.e., the mortality in outmigrating wild salmon smolt is estimated to be more than 30% due to salmon lice (*Lepeophtheirus salmonis*) infestation.

In 2022, the mortality rate in PO4 for farmed salmon was 22% and 14.5% for rainbow trout (Sommerset et al. 2023).

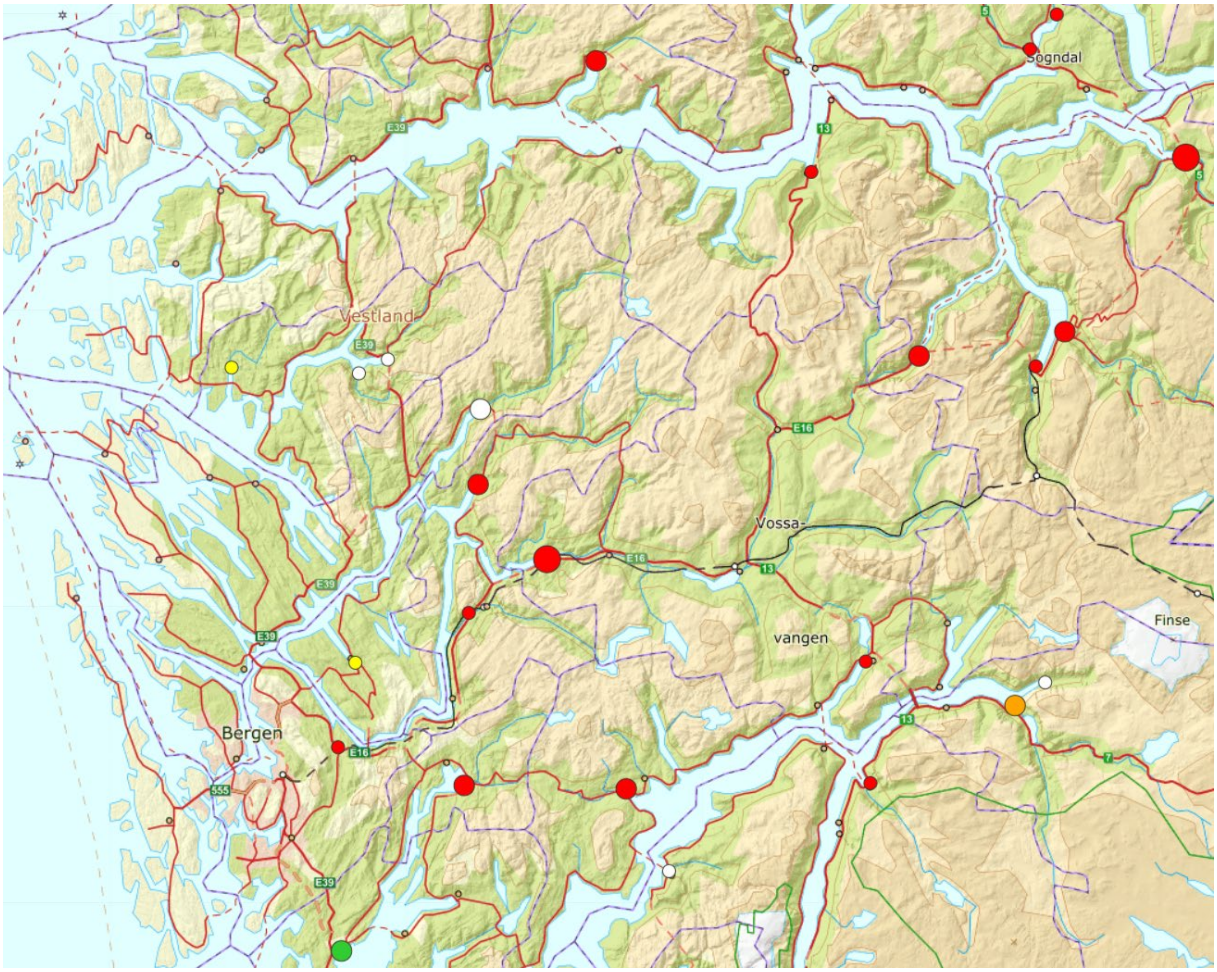

Figure 5: Status of Atlantic salmon in rivers from the Hardangerfjorden in the south to the Sognefjorden in the north, according to the Quality Standard for Atlantic salmon (Kvalitetsnorm for ville bestander av atlantisk laks (Salmo salar) - Lovdata) and assessed by VRL (2021). Green = Good status, Yellow = Moderate status, Orange = Poor status, Red = Very poor status, White = Not assessed. The size of the symbols in the map is proportional to the river's spawning target (in kg female biomass) from the largest symbol (> 2000 kg) to 200-2000 kg to < 200 kg for the smallest symbol. Average body size of female salmon in the area shown vary from 2.5 kg to 7 kg and the number of spawning wild salmon varies from less than 50 (R. Kinso) to more than 800 (R. Lærdalselva). Map extracted from www.vitenskapsradet.no

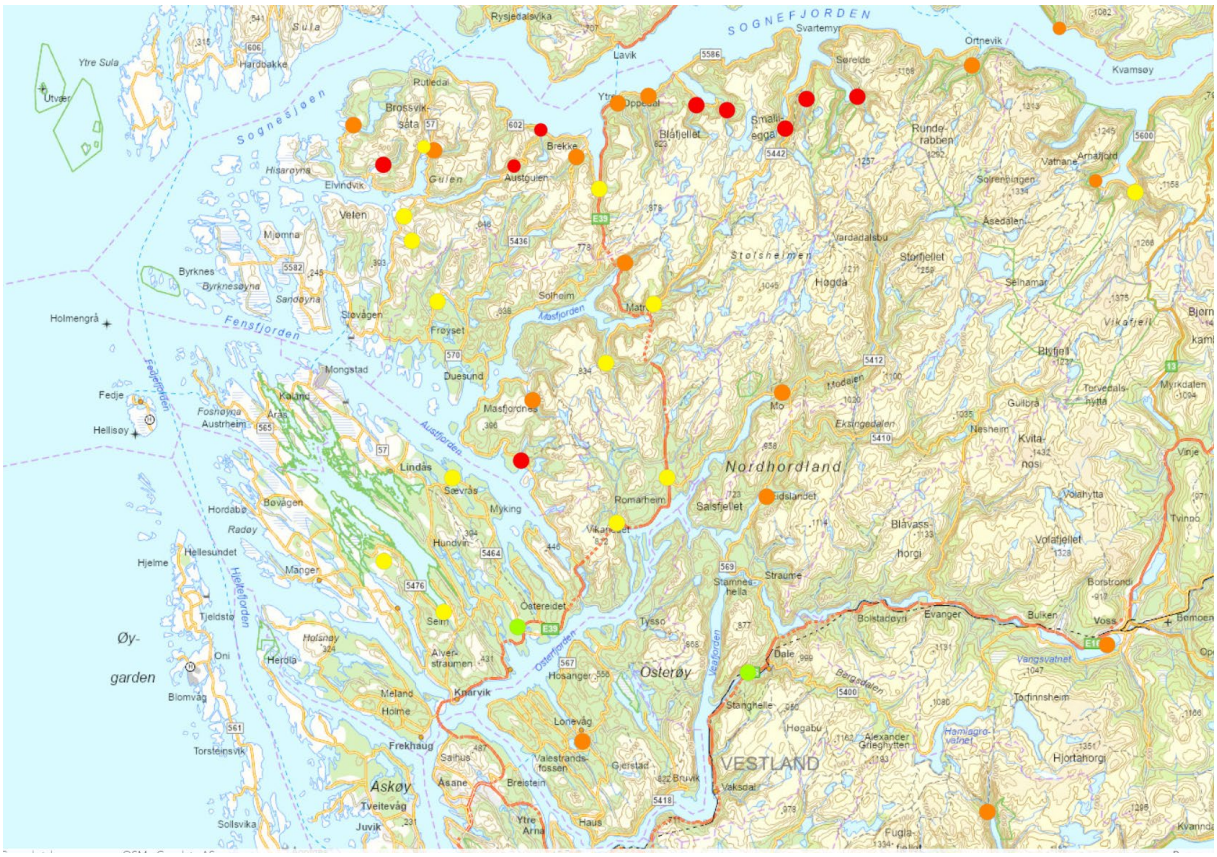

Figure 6: Status of sea trout populations in rivers from Osterøy in the south to the Sognefjorden in the north. Green = good status, Yellow = moderate status, Orange = Poor status, and Red = Very poor status according to VRL (2022). Map extracted from www.vitenskapsradet.no. Areas encircled with green colour are marine protection areas.

The status of most wild Atlantic salmon populations in the region where Matre is situated is classified as very poor (Figure 5) in the classification system of the Norwegian Scientific Committee for Wild Atlantic Salmon (VRL 2021). The classification system follows a Regulation (Quality Standard for wild Atlantic salmon populations) that emanates from the Nature Diversity Act. In the Quality Standard, both quantitative criteria relative to river-specific spawning target and harvestable surplus, and qualitative criteria based on estimates of farmed to wild salmon genetic introgression, are considered.

The status of wild Atlantic salmon populations is also classified as very poor in the nearest rivers designated as National Salmon Rivers. The two closest National Salmon Fjords to Matre are the fjords around Osterøy (near River Vosso) to the south and the inner half of the Sognefjorden to the north (including rivers Årøyelva, Vikja, and Lærdalselva, among others). Sea cages are not allowed in National Salmon Fjords, whereas land-based production is permitted.

The same committee classified the population status of the congeneric sea trout (*Salmo trutta*) in the same region (VRL 2022). Also, for this species, most populations were classified as having a very poor status (Figure 6).

The Matre Aquaculture Research Station is situated in the Masfjorden, which empties into the larger Fensfjorden. This area is relatively densely populated with fish farms (Figure 7).

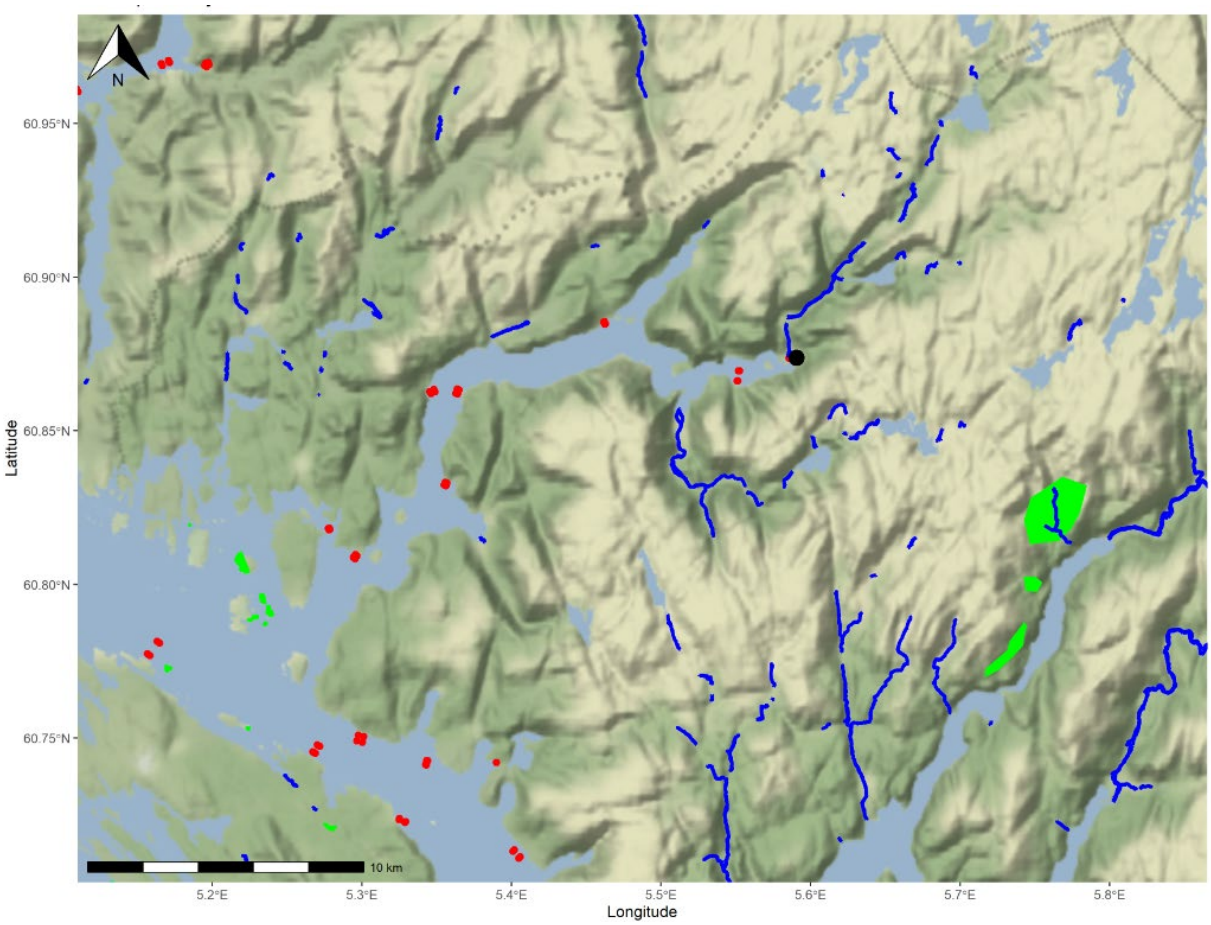

Figure 7: Map of Masfjorden with IMR Aquaculture Research Station at Matre indicated by a black symbol and fish farming localities in Masfjorden and the neighbouring Fensfjorden indicated by red dots.

In week 36 of September 2023, there was a protection zone caused by ISA (Infectious Salmon Anemia) northwest of Sotra (Figure 8) and two ISA surveillance zones between the protection zone and the outlet of Masfjorden into Fensfjorden. A protection zone is established around sites with ISA detected, whereas surveillance zones are areas where increased monitoring efforts take place.

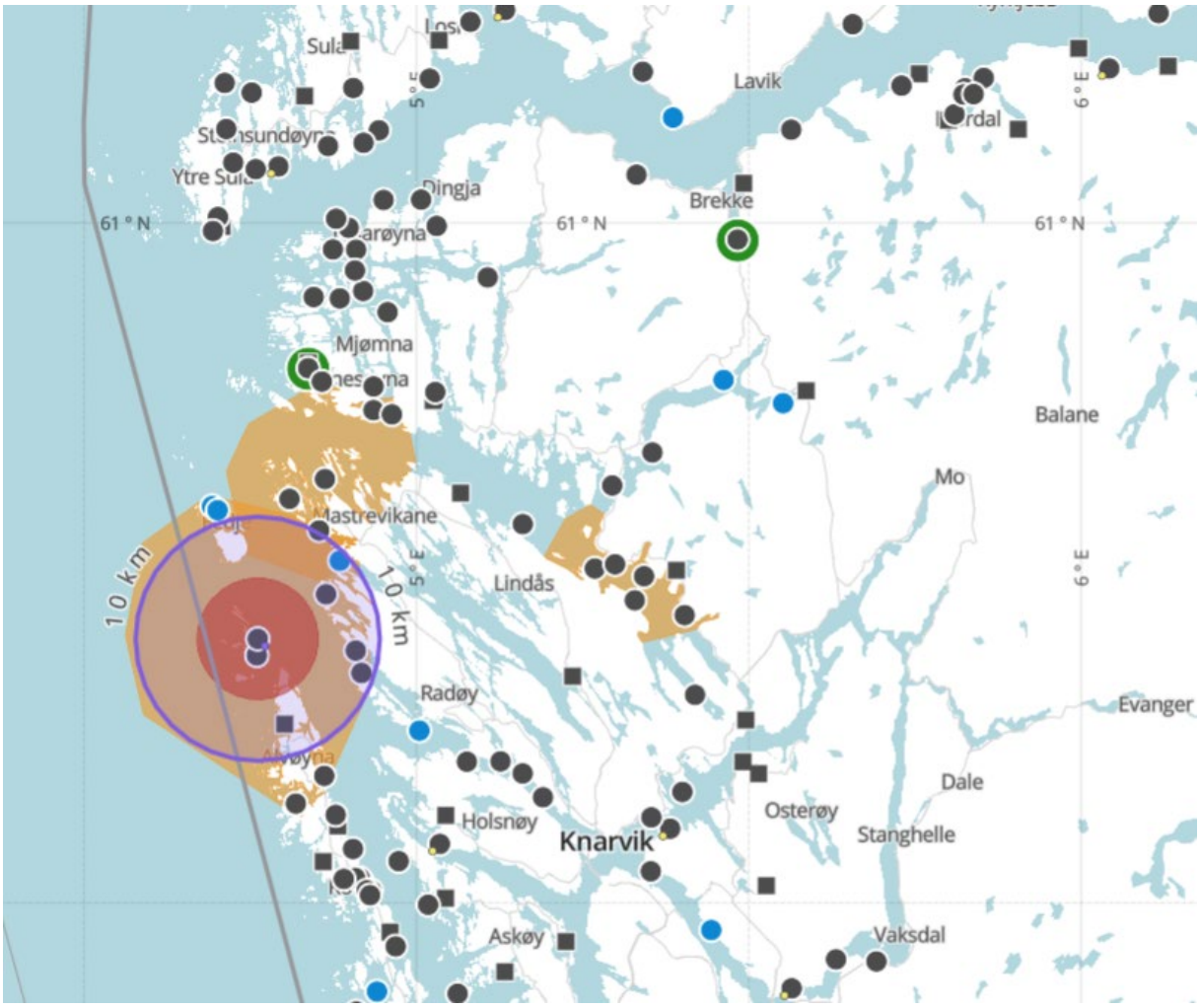

Figure 8: Map of the Norwegian coast from Sotra and Askøy in the south to the outer part of the Sognefjorden in the north, with Matre and Smørdalen indicated by a black square next to a blue dot. The red area indicates an ISA (Infectious Salmon Anemia) protection zone with the blue line indicating the surrounding area within 10 km by week 36, September 2023. Orange fields indicate ISA surveillance zones, two of which overlap with migration routes of wild salmonids from the Masfjorden (Map from Barentswatch.no, September 2023).

2.1.3 Experimental environment

The 303 F1 VIRGIN salmon and 485 sibling WT control salmon shall be released as autumn smolts into four net pens. F1 VIRGIN salmon and controls will be reared in a common garden setup in the four small net pens with 197 individuals per net pen. PIT antennas at three depths in the larger net pen (12 x 12 m) will be used to estimate the swimming depth of all individuals. All individuals will be measured for length and weight at seawater transfer, after 5 and 10 months, and at slaughter after 18 months to compare growth patterns between F1 VIRGIN salmon and controls. Fish will also be scored for welfare indicators at these sampling time points. Dead fish will be collected daily, and environmental conditions (temperature, salinity, and oxygen) will be monitored continuously during the rearing period.

2.2 Choice of comparators

2.2.1 Choice of comparators for ERA of GM fish

The EFSA Guidance Document on the ERA of GM animals (EFSA 2013) suggests that ERA of GM fish should compare the GM fish to:

1. its non-GM source progenitor line,
2. one or more populations of wild fish within the same species,
3. one or more populations of wild fish species exploiting a similar niche as the GM fish, and
4. aquaculture lines of the same species as the GM fish.

This list is meant to cover cases where it is proposed to release GM fish to the environment.

In the present application, the intended release is of 303 F1 VIRGIN and 485 WT control fish into a fish farm with double net pens. In such cases, EFSA (2013) states that "In addition to the non-GM line, applicants should use at least one wild population as comparator where the risk assessment has predicted that escape into environments occupied by wild types is a possibility".

The present application suggests that for analyses of performance and welfare in the net pen experiment, the WT siblings of the F1 VIRGIN salmon are the comparators of choice ("conventional counterpart"). The molecular information from the crosses that gave rise to both the 303 F1 VIRGIN salmon and the 485 WT control group is insufficient to assess the value of this comparator. The two groups are referred to as siblings. They are the result of crosses between a limited number of parents (two males crossed with four females) and genetic analyses of fin-clips of 2200 individuals to sort out fish that are homozygote knockouts (303) and another group that are homozygote wildtype (485) in the *dnd* gene. There is no information on how many of the maximum eight families that are represented in the total test material, or whether the same families are equally present in either group (F1 VIRGIN vs WT control group).

VKM assesses that the few families studied, and the selection of both groups from CRISPR-generated parents, might introduce non-random genetic similarities among the test groups that make the results of the proposed tests of limited value for salmon in general. As a minimum, both groups should also be compared with conventional farmed fish in the same experiment (by introducing a third group).

For the ERA, VKM also uses information from experiments with escaped farmed and wild Atlantic salmon, where appropriate.

2.3 Animal trials for comparative analysis - experimental design and statistical analysis

2.3.1 Experimental design and statistics

The 303 F1 VIRGIN salmon and 485 wildtype controls are the offspring of two F0 VIRGIN rescued males and four F0 VIRGIN rescued females. Each male was crossed with the same four females creating eight offspring groups that were lumped at startfeeding. The limited number of F0 VIRGIN rescued salmon (two males by four females) producing the 303 F1 VIRGIN salmon and their 485 wildtype sibling controls, together with missing information on their representation among the fish in the different experimental groups, introduces risk of confounding of e.g., genetic effects of male parent and effects of introduced mutation (sterility). This may introduce biased estimates of effects and hence reduce the validity of the results.

For genes that are characterized by many variants (alleles) in Atlantic salmon, such as the immune response genes Mhc1 and Mhc2, two males and four females cannot possibly harbour all the allele variants that are present in a population (Grimholt et al. 2003, Kjøglum et al. 2006, 2008). With few parents, most of the rare alleles in farmed salmon will be lost. We do not know, however, to what extent this makes the F1 VIRGIN and WT controls different from conventional farmed salmon.

In written answers to questions posed by VKM, the applicant states that "Since VIRGIN fish only has a mutation in the *dnd* gene, it is reasonable to compare it to a comparator which has similar genetic setup. In this case we have chosen WT siblings from the same strains and families used in the crossing of VIRGIN parents." There is no statement in the application about the number of individuals in each family. VKM has asked about information on the family structure of the test and control groups. However, no quantitative information has been given.

The application does not state how the interaction between infectious agents and F1 VIRGIN salmon and WT controls will be studied. On the contrary, the study will be terminated if a disease outbreak occurs.

2.4 Conclusions on cross-cutting considerations

The ambition of the project is to study how VIRGIN salmon perform and behave in sea cages and to test if they are like conventional farmed salmon, by housing the salmon in a realistic environment for sea-cage aquaculture of Atlantic salmon.

The experimental design is based on a very limited set of parents. The likelihood of genetic confounding increases when only two males by four females are used to produce the 303 F1 VIRGIN salmon and their 485 wildtype sibling controls. In addition, information about family representation among the fish in the different experimental groups is missing. This may introduce biased estimates of effects and hence reduce the validity of the results.

Moreover, EFSA (2013) guidelines suggest that at least one wild population be used as comparator for experiments where the risk assessment has predicted that escape into environments occupied by wild populations is a possibility.

2.5 Methodology for environmental risk assessment

For the environmental risk assessment, VKM has used a semi-quantitative approach. The overall risk is the product of the magnitude of the consequences of the event and the likelihood that the event will occur, as judged by the project-group experts. The resulting risks are thus defined as presented in Figure 9.

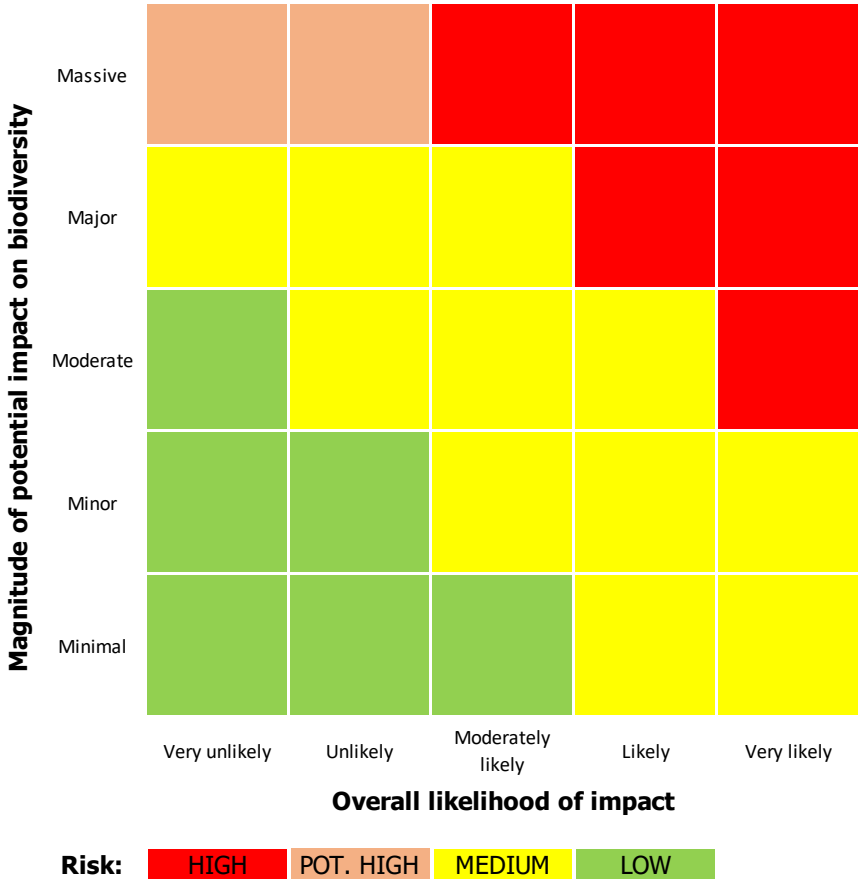

Figure 9: The conclusion of the risk assessments (low, moderate, potentially high or high) is based on the overall likelihood of the impact and the magnitude of the potential consequences of that impact on Norwegian biodiversity. The Potentially High Risk covers situations where the risk assessment changes outcome from occurring only within the lifetime of released fish to affecting several generations.

The VKM risk assessment varies based on whether fish escape from the experiment and whether any of them are not sterile. The resulting risk for a massive impact occurring with low likelihood is termed 'potentially high risk' (Figure 9). Such a situation could occur if fertile escaped individuals from the experiment carried sterility alleles to wild populations (see Chapter 3 and 4).

In order to provide justification of why a rating is given in the risk assessment, the project group used ratings and adapted versions of the descriptors from Appendix E in (EFSA Panel on Plant Health (PLH) 2015). A description of the ratings used for this risk assessment can be found in Tables 2.5-1–2.5-3.

Table 2.5-1 Ratings used for the assessment of the magnitude of the impact.

Rating	Descriptors
Minimal	No known impact on local populations
Minor	Potential impact on local populations
Moderate	Impact may cause reduction in viability and adaptability of local populations
Major	Impact may cause reductions in local populations and populations further away
Massive	Impact may cause reductions in local populations and populations further away, and affect many generations

Table 2.5-2 Ratings used for the likelihood of impact.

Rating	**Descriptors**
Very unlikely	Negative consequences would be expected to occur with a likelihood of 0-5%
Unlikely	Negative consequences would be expected to occur with a likelihood of 5-10%
Moderately likely	Negative consequences would be expected to occur with a likelihood of 10-50%
Likely	Negative consequences would be expected to occur with a likelihood of 50-75%
Very likely	Negative consequences would be expected to occur with a likelihood of 75-100%

Table 2.5-3 Ratings used for describing the level of uncertainty.

Rating	**Descriptors**
High	Available information on the topic is limited, and mostly expert judgements are used.
Medium	Some published information exists on the topic, but expert judgements are still used.
Low	There is sufficient published information, and expert judgements are in concurrence.

3 Environmental risk assessment (ERA)

3.1 A staged approach to environmental risk assessment

In the risk assessment, we have identified several hazards of which some can come into play without the fish entering the surrounding environment, while others can only become a reality should the fish escape from the experiment to the natural environment.

In the case of no fish escaping to the wild from the research trial, the ERA shall only consider impacts from confined aquaculture facilities holding of GM fish (Box 1 in Figure 10). In this case, the risk assessment is limited to hazards caused by infectious disease agents or by handling of fish by humans. These hazards are treated in chapters 4.3 to 4.4. Other potential hazards, like those caused by excrements or feed remains, are not considered to be different from the comparator and thus not assessed further.

If fish escape from the experiment, but are not capable of reproduction, the ERA shall consider ecological impacts of GM fish in the natural environment (Box 2 in Figure 10). This includes escaping to the fjord environment of the Smørdalen facility and further spread to the ocean and/or to neighboring rivers and other places along the coast. Ecological effects of escapees can be competition, predation and displacement of wild salmon. In addition, there may be additional epidemiological effects if fish that escape from the experiment carry infectious disease agents.

If some of the fish that escape from the experiment are capable of reproduction, the EFSA (2013) staged approach distinguishes between three scenarios of genetic (as well as ecological and epidemiological) effects. The first relates to the effects of escaped fertile fish interbreeding among themselves but not with wild populations (Box 3 in Figure 10), the second relates to escaped fertile fish interbreeding with wild populations of the same or related species in their current range (Box 4 in Figure 10) and the third is a combination of escaped fertile fish being established in the current range of the species as well as establishing new, feral populations (Box 5 in Figure 10).

3.1.1 Salmon escaping from aquaculture and factors influencing this event

During the last ten years, the average number of Atlantic salmon reported to escape from Norwegian salmon farms each year is about 140 000 salmon (Directorate of Fisheries). The number of salmon reported as escaped by fish farmers is likely an underestimate of the real number (Wennevik et al. 2023), but the extent of underreporting is not known. The livestock of farmed Atlantic salmon in aquaculture pens as reported 31 December each year increased from 379 million fish in 2013 to 436 million fish in 2022 (Directorate of Fisheries). *Hence, farmed salmon escape from fish farms, but in small numbers compared to the total production of farmed salmon. The number of escapes may vary from a few farmed salmon to entire net pens or (in the worst case) all fish held in a fish farm.*

There are several reasons why fish escape from fish farms. Salmonids primarily escape after structural failures of containment equipment (Jensen et al. 2010). In 2010-2018, most registered Atlantic salmon escapees came from sea-based fish farms (92%), while 7% were from land-based facilities and 1% from transportation between sites (Føre & Thorvaldsen 2021). Most escape incidents were directly caused by technological factors, with holes in the net as the most common cause of escape.

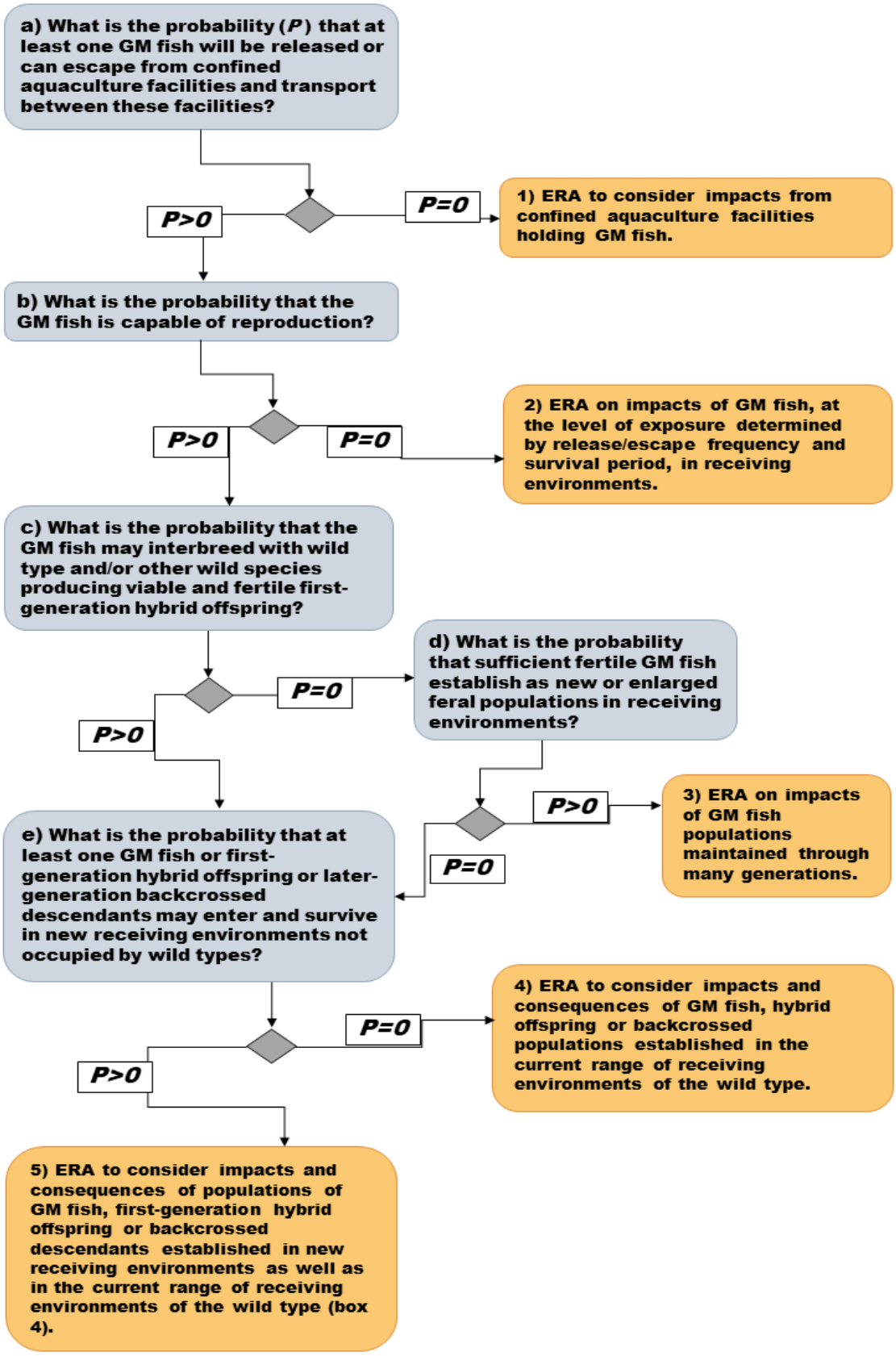

Figure 10. Staged approach for risk assessment of genetically modified fish (adopted from (EFSA, 2013).

Extreme weather, accidents during transfer or handling of fish, or handling of weights and net prior to delousing have been associated with increased probability of escape incidents (Directorate of Fisheries). Heavy storms can also cause equipment breakdowns that allow fish to escape (Directorate of Fisheries). Analysis of contributing causes by Føre and Thorvaldsen (2021) show that half of the fish that escaped through holes were caused by the weight system, and that wear from bottom ring chain and handling of weights were important factors. Another significant fraction of the holes was caused by conflict with or damage to main components or auxiliary equipment. The category main components include conflict with or damage to mooring system, feed barge, cage collar and issues regarding net cage structure (defects) and handling of the net.

The net pens used in the present study will not be standard net pens but include four smaller cages (5x5 m) tied and anchored within a larger net (12x12 m). In the application, it is described that this system is used occasionally at Matre, but not normally at this experimental site. The application refers to use on one occasion in 2022 at another site five km away when 4.2% of the fish had clear fin damage, 1.5% clear scale loss, 0.22% clear eye opacity, and 0.22% clearly short operculum. *Hence, it is likely that some fish kept in these net pens will have injuries from being kept in this type of pens.*

We conclude that like in all net pens in the sea, there is a risk that (some or all of) the experimental fish can escape from these pens into nature if accidents occur during operation and extreme weather. The system with pens within a pen is not a commonly used system in commercial aquaculture and is previously not used at the site and may therefore be prone to unforeseen accidents. A double pen may add security if one of the net layers is damaged. However, the unusual set up with small pens being tied to the large pen and anchors hanging from the small pens within the large pen may increase the risk that the anchors/nets can entangle in each other and damage the nets. This set up may possibly also imply a different/extra drag during windy conditions and currents. Hence, this may increase the risk of escape episodes compared to standard pens used in fish farming. It is not clear how well this set up can be trusted during extreme weather episodes. The application states that in the case of storms that may threaten the integrity of these facilitates, they may transfer the fish to on land sea cages. Such transport operations, particularly during extreme weather, can increase the risk of farmed fish escaping.

Mesh size is relevant both for the assessment of escape risk and of the fish being injured and therefore more prone to infections while they stay in the pen, which again impacts the health risk they impose to wild fish should they escape. VKM has asked the applicant twice for details on the mesh sizes that will be used in the experiment, but not received information on this. In the application it is stated that “We use mesh size in relation to fish size in accordance with the regulations applicable to farmed fish (), which means that the mesh size will depend on the size of the fish at release into the sea cage.” The regulation does not specify which mesh sizes must be used with the different fish sizes, but §37 says *Maskeåpning i notpose og notpanel skal være tilpasset fiskens størrelse, slik at fisken ikke kan slippe gjennom notpose eller notpanel*. This means that not only the mean size of the released fish is of interest, but more importantly, the smallest fish size. The likelihood of fish escaping can also increase if the inner and outer net pens are adapted by changing the mesh size as the fish grow. *Overall, VKM assesses that it is uncertain whether the likelihood of fish escaping from the experimental pens differs from the likelihood of escaping from standard net pens used in fish farming.*

3.1.2 Ecological interactions between escaped farmed salmon and wild salmon and factors influencing this event

During the nine last years, the average proportion of escaped farmed salmon in angling catches in Norwegian monitored rivers during summer varied between 1% and 7% (Wennevik et al. 2023). During monitoring in the rivers in the autumn shortly before the spawning period, the proportion of escaped farmed salmon varied between 4% and almost 12% (Wennevik et al. 2023). Hence, some escaped farmed salmon survive and enter the rivers before the spawning period of wild salmon.

There are several studies of behaviour of escaped farmed salmon based on simulated escape events (Heggberget et al. 1993, Hansen 2006, Skilbrei et al. 2009, 2010, 2015, Hansen & Youngson 2010, Skilbrei 2010a,b, 2013, Skilbrei & Jørgensen 2010, Chittenden et al. 2011, Solem et al. 2012a,b, Uglem et al. 2013, Skilbrei et al. 2015). These studies show that farmed smolts that escape in the spring or post-smolts escaping in the following summer may migrate to the ocean like wild salmon and return to rivers after one to several years in the ocean. Some of these return to rivers near the site they escape from, whereas others may spread over large areas and be recaptured in all parts of the country.

Post-smolts that escape in the autumn may have lost the motivation to migrate to the ocean and may to a larger extent remain in the area where they escaped during the autumn and following winter. When larger salmon escape, they seem to spread from the escape area during a few days or weeks, independent of time of the year the escape occurs. These fish seem not to have a homing instinct to the area where they escaped from, and they may enter rivers hundreds of kilometres away from the escape area (Hansen 2006, Skilbrei et al. 2015). Escaped salmon from Norway have been recaptured in other countries, like Russia, Sweden, Finland, and United Kingdom. When adult salmon escape in the spring, summer, or autumn, they may stay close to the area where they escaped from and enter rivers close by. These salmon seem to have higher survival rates than those escaping in the autumn. Many of the studies on escaped salmon are based on recaptures of tagged fish reported by fishers, whereas some are based on electronic tagging. Hence the behaviour of recaptured or recorded fish is known from studies during different life stages and times of the year, but the absolute survival rate of the escaped fish is not known.

Several of the studies on behaviour of escaped farmed salmon that are based on simulated escape events are performed from the research station at Matre (Skilbrei 2010a,b, 2013, Skilbrei et al. 2015). These studies revealed recaptures ranging from Nordland to Telemark. Even though most of the recaptures were near the release site, the geographic area affected by escapes from this location could be extensive. Skilbrei et al. (2010) studied the behaviour of adult salmon (average body mass 54-72 cm, 2.8-4.3 kg) after simulated escape events throughout the year and found that the escaped fish spread quickly in the fjord and were on average 5-7 km from the release site after one day, 9-12 km after two days and spread over an area of 500 km^2 after one week. After three weeks, 40% of the fish were still in the fjord, whereas after 7 weeks, all fish had left the fjord. The fish dove to more than 15 m depth during the first hours and days after release, often down to 50-80 meters depth, but later returned to near surface layers at 0-4 m (Skilbrei et al. 2009). A similar vertical behavioural pattern was recorded after a simulated escape of adult salmon by Chittenden et al. (2011). This swimming at deeper depths during the first hours and days after release reduces the efficiency of attempts to recapture escaped salmon with nets.

Escaped farmed salmon are recorded in several feeding areas of wild Atlantic salmon in the Northeast Atlantic Ocean, likely originating from Norwegian fish farms (Hansen et al. 1993, Hansen and Jacobsen 1998, 2003, Hansen et al. 1999). Escaped farmed salmon in the ocean show similar feeding and have a similar body condition as the wild salmon (Jacobsen and Hansen 2001). Common food sources of salmon in the ocean are amphipods (*Themisto*), euphausiids and mesopelagic shrimps and various mesopelagic fish, such as lantern fishes, pearlsides, and barracudinas. Salmon smolt may feed on zooplankton and fish larvae, whereas larger salmon may feed on herring, blue whiting and mackerel. There is likely competition in the ocean between salmon and similar-sized marine species. Due to their low abundance, Atlantic salmon are not viewed as a dominant species in the ocean.

Escaped farmed salmon often enter the rivers later in the season than the wild salmon, and the proportions in monitoring in the autumn are often higher than during the angling season in the summer (Wennevik et al. 2023 and references therein). Escaped farmed salmon often distribute themselves high up in the rivers, particularly in rivers without waterfalls that are difficult to pass.

The long-term survival of escaped farmed salmon has not been studied as far as we know, since the long-term behavioural studies are mainly based on reports of recaptured fish by anglers. Only a small proportion of the fish will be recaptured and reported by anglers, and the fate of surviving but not recaptured fish is not known. In the application, it is estimated based on published recapture rates that 0-1.1% of the fish are expected to survive, assuming that recapture rate equals survival rate. This is not a correct assumption. The real survival rates are not known but are inevitably larger than the reported recapture rates in the different studies. Moreover, escape events are known where a considerable proportion of the escaped farmed salmon moves directly into rivers (Wennevik et al. 2023).

How the immature experimental salmon in this study may behave and survive if they escape is not studied and not known. If they do not mature and take part in reproduction, their direct ecological effect is limited to their lifetime (see Box 2 in Figure 10). It must be expected that they can distribute widely, like other escaped farmed salmon, in fjords and to feeding areas of wild salmon in the Atlantic Ocean. Even those that do not mature may enter rivers, as is known for immature escaped farmed salmon (Lund 1998, Wennevik et al. 2023). It should also be anticipated that the experimental fish may spread to both nearby and distant rivers. This means that these fish can interact with wild fish and fish in other aquaculture farms and potentially spread infections. Since they are not externally marked, this can go unnoticed. It is not expected that immature salmon take part in the spawning activity of wild fish.

The application states that standard procedures for capturing escaped salmon will be followed in the case of an escape event. No special attempts to recapture the fish will be made. Dempster et al. (2018) concluded that recapture of escaped fish is broadly ineffective in marine habitats, with rare exception, and that recapture efforts may harm local wild fish that are caught as bycatch. The low recapture rates are due to the escaped fish spreading rapidly over large areas, and that they are moving relatively deep in the water after escaping.

During the common life cycle of Atlantic salmon in Norway (as well as in Iceland, Canada, and Barents Sea populations in Russia), all individuals return from the sea in spring and summer, and they mature

and spawn in the coming fall or early winter (Klemetsen et al. 2003). Typically, large multi-sea-winter salmon enter a river first and smaller one-sea-winter fish enter later in the season.

Escaped farmed salmon enter rivers in summer and autumn and typically later than wild salmon. Lund (1998) found that among escaped farmed salmon males caught in 10-30 rivers in the autumn of the years 1989-1997, 6% (range, 0 to 21%) were immature. For females, the corresponding proportion was 12% (range, 0 to 36%). In the current surveillance program for escaped farmed salmon in Norway, Wennevik et al. (2023) found 38% immature individuals (range, 11-51%) for the years 2014-2022. In Canada, Bradbury et al. (2020a) found 37% immature individuals among escaped farmed salmon entering fish traps in Newfoundland rivers. In a recent study from a fish trap in the River Etne, Madhun et al. (2023) found that among 616 escapees collected during 2014-2018, 45% were sexually immature and 55% were sexually mature. Most of the escapees were categorized as recent escapees (90%), meaning that they had not been on the run for long before entering the river. Among the escapees that had stayed in the sea for a prolonged period before entering the Etne fish trap, only 4% were immature.

Immature escaped farmed salmon tend to be smaller in body size than mature escaped farmed salmon (Wennevik et al. 2023 and references therein). They are often found in the lower part of rivers and have stayed for a shorter period at sea after escape than mature escaped farmed salmon. In an opportunistic study in the Hardangerfjorden from mid-August to early September 2012, Madhun et al. (2015) found that all but one of 59 small farmed Atlantic salmon (average weight 415 gram) were immature and that 21 of them had entered the local River Steinsdalselva.

In a Canadian river, Carr et al. (1997) found that immature farmed salmon made shorter river migrations than sexually mature farmed salmon, but also that they stayed longer in the river without moving downstream. They suggested that this could have ecological implications in the river but to our knowledge, did not follow this up.

Wild salmon are not known to feed extensively after entering the river before spawning. Kelts (i.e., spent salmon) can resume feeding already in fresh water during their return to the sea after spawning (Thorstad et al. 2011) but feeding in this freshwater phase is likely not common (Halttunen et al. 2013). In some individuals, the return to the sea to resume feeding occurs immediately after spawning or during the following winter, particularly in small males. Halttunen et al. (2013) found that individuals with low energy reserves migrated to the sea early after spawning, whereas individuals with greater energy reserves spent several months in the river before migrating to the sea.

The highest ecological effect of immature individuals from the proposed experiment on wild fish populations would likely occur if immature experimental fish entered local rivers and started feeding on juvenile salmon or trout. Immature experimental fish may reach a large body size and may be larger than immature escaped farmed salmon. Feeding in fresh water is likely a behaviour that depends on the physiological status of the immature fish, as well as the costs and benefits of feeding on juvenile salmonids. Juvenile salmonids may be easier prey during their outward migration in spring, and when precocious males engage in spawning in autumn, than at other times of the year.

As far as we know, no experiments have been performed that could inform how F1 VIRGIN salmon could interact with wild salmon if they stayed immature throughout their lifetime.

3.1.3 Potential interactions if GM-fish are not sterile and factors influencing this event

Based on the information provided in the application, VKM concludes (chapter 1.3) that there is a possibility that an unknown number of potentially fertile heterozygous individuals among the F1 VIRGIN salmon (n = 303), and also a possibility that an unknown number of the wildtype controls (n = 485) may in fact be fertile heterozygous fish, carrying a mutated *dnd*-allele. We must therefore consider effects of escaped salmon from the research trials interbreeding with themselves, with other farmed escapes, or with wild salmon (see boxes 3 to 5 in Figure 10).

The WT/WT controls of the experiment are intended to be both physiologically and genetically fertile, like conventional farmed salmon. We expect the behaviour and potential reproduction of fertile WT/WT individuals in the wild, to resemble escaped farmed salmon in the wild. If they escape early in life, farmed females show a reproductive success that is non-significantly different to that of wild salmon (Lura 1995, Fleming et al. 1997). Escaped farmed males, on the other hand, have significantly reduced reproductive success compared with wild males, and their success decreases with increased spawner density (Fleming et al. 1997). If escaping late in life, the reproductive success of farmed females may be more than halved relative to that of wild females (Fleming et al. 1996, 2000, Lura 1995) and may be even more depressed among males.

Offspring of farmed escapes show variable survival in comparison with offspring of wild salmon. They may show competitive advantage at some life stages but in general show reduced survival both in the freshwater and seawater life stages (Fleming et al. 2000, McGinnity et al. 2003, Skaala et al. 2012, 2019). Experimental and observational studies demonstrate that wild populations that are genetically introgressed with farmed fish show changes in many traits associated with fitness. These traits include growth rate in fresh- and seawater, age at smoltification and maturation, body size at maturity, egg size, longevity, and phenology of smolt outmigration and return of adult fish (Glover et al. 2017, Bolstad et al. 2017, 2021, Skaala et al. 2019, Besnier et al. 2022).

Genetic introgression of farmed to wild fish in Norwegian rivers has been demonstrated in 1/3 of the salmon populations and indicated in another 1/3 of the populations (Diserud et al. 2020, 2022). Introgression most often occur in rivers near intensive aquaculture where a majority of escaped farmed salmon is found. In the River Matreelva, close to the proposed experiment, significant genetic introgression has been found in a sample of 17 juvenile individuals from 2012 (Diserud et el. 2020). Significant genetic introgression has also been found in the closest National Salmon Rivers to the south (River Vosso) and to the north (rivers Årøy, Lærdal, and Vikja in the Sognefjorden) of the proposed study site (Diserud et al. 2020).

In conclusion, we expect the WT/WT controls of the experiment to have the same genetic effects as conventional farmed salmon if they escape. There may be some additional uncertainty resulting from whether or not all of the 485 controls are correctly classified (see chapter 1.3).

The genetic effects of F1 VIRGIN salmon, if they were not 100% sterile, are not known. We can only speculate on this. The application states that “VIRGIN fish are genetically sterile, so it is not expected

that they will be able to crossbreed with wild populations of salmon. If a mutation in the *dnd* gene were to enter wild fish populations, it is likely to quickly disappear since there is no selection for traits that make the fish sterile. Having mutations in the *dnd* gene means poor fitness for both farmed fish and wild fish since fertility will decrease." It is unclear if the applicant by this means that the *dnd* gene could enter a wild population by spawning of fish escaping from this experiment, or only by spontaneous mutation in (any) wild salmon.

Potentially fertile individuals among the 303 F1 VIRGIN salmon are likely heterozygotes with one fertile (WT) and one sterile (*dnd*-KO) allele. We assume the knock-out alleles to be recessive since they do not code for germ cell-formation, whereas a fertile allele does. Successful crosses with wild fish should then result in ½ wildtype individuals and ½ heterozygote individuals. The knock-out alleles are recessive in heterozygotes and would be hidden from selection in the population where they enter (Hedrick 1983). Although positive selection for a mutated *dnd* allele is unlikely, further spread of the sterility (knockout) allele would be possible in situations where escaped individual(s) produces many offspring. A female Atlantic salmon produces 1300-2000 eggs per kg body weight (Klemetsen et al. 2003) and a sterility allele might enter a small Atlantic salmon population at a high frequency.

If two heterozygote (knockout/wildtype) individuals from the experiment interbred, it would be expected that ¼ of the offspring were knockout/knockout homozygotes, ½ were heterozygotes and ¼ were wildtype/wildtype homozygotes. This would incur a penalty (25% sterile individuals) among the offspring of this cross. The same cross would be likely to occur in the second offspring generation in a wild population receiving heterozygotes from the experiment, in which case the penalty would be taken by the wild population by recessive homozygotes competing for food and space in the river as juveniles but not contributing to spawning.

If heterozygote (knockout/wildtype) individuals from the experiment leave offspring in rivers, a short-cut to further introgression of sterility alleles into the wild populations can occur through the spawning of precocious male salmon, that is, males that attain sexual maturity before migration to sea (Weir et al. 2005).

In chapter 4, we present a numerical example of how long a sterility allele may persist in a wild population.

4 Results of the ERA

4.1 Persistence and invasiveness of GM-fish, including vertical gene transfer

The survival and spread of escaped farmed fish depend on the life stage and time of year of the escape, and their effect in the wild depends on the status of the populations in rivers the escapees enter. In the region where the experiments shall take place in aquaculture zone PO4 of Vestland county, the status is poor for most populations of Atlantic salmon (Figure 5) and for anadromous (sea) brown trout (Figure 6) (VRL 2021, 2022).

If 100% of the F1 VIRGIN salmon are sterile (and immature as long as they live), their impact cannot be caused by interbreeding. Any effect is therefore ecological, occurring by competition, predation or displacement, or epidemiological by increasing the transmission of infectious diseases, and only indirectly genetic by either population reduction (loss of genetic variation) or novel selection pressures (Bradbury et al. 2020b).

To our knowledge, there is no information on gene-edited sterile salmon in the wild. We know little about their biology apart from the comparison in a land-based facility at Matre, where the growth rate of F0 VIRGIN salmon was similar or slightly less than that of control fish (Kleppe et al. 2022) and their longevity was 5 years.

If all experimental fish were sterile, persistence of GM-fish in the environment would not occur beyond their lifetime. The research trial will end in February 2025 and remaining F1 VIRGIN salmon euthanized and incinerated. If they escape before this, they may live longer than 5 years in the wild.

Vertical gene transfer through the spawning of fertile experimental fish, is the sole means for persistence beyond the lifetime of the experimental fish. The EFSA (2013) guidelines distinguish between spawning of GM-fish in new environments (outside the range of the species; Box 3 in Figure 10) and spawning with wild conspecific populations within the species range (Box 4 in Figure 10). During recent decades, salmon spawning in small creeks has been a more common phenomenon in Norway than before the turn of the century. In Vestland county, Pulg et al. (2020) found Atlantic salmon juveniles in several creeks and small rivers, including one creek on the island of Sotra, which looked like being established by escaped farmed salmon. It is therefore possible that similar populations could be established by fertile experimental fish breeding among themselves in odd localities.

4.1.1 Hazard identification

VKM has identified the following hazards if experimental fish escape from the net pens to the environment:

If physiologically and genetically fertile, the escapees (wild-type controls) can spawn successfully with wild Atlantic salmon in rivers and lead to introgression of farmed salmon into wild populations. They can also spawn with brown trout and lead to the formation of interspecific hybrids. Both of these hazards are known and will be similar to hazards described for escaped farmed salmon above.

If physiologically and genetically sterile, the escapees (F1 VIRGIN salmon) will be sexually immature for life. In that case, hazards are not directly genetic (through vertical transfer) but they may be

indirectly genetic through the effects of other interactions like predation on juvenile salmon or brown trout (see also Chapter 4.3 on Fish pathogens, whose impact may increase with escapees but may also impact other farmed and wild salmon without escape).

If physiologically fertile but with genetically sterile *dnd* allelic variants, the escapees (likely heterozygote knockout/wildtype salmon) can spawn successfully and transfer genetic sterility to salmon offspring in the rivers. This is a hazard that is not known from previous experience and its impact may vary according to the number and body size of heterozygote (sterile/fertile) salmon that escape and their reproductive success. In this case, the risk assessment must also include the effects of the offspring that inherited the sterility allele and could potentially spread sterility alleles to other populations.

When experimental fish escape from net pens, they enter a fjord where the impact is likely to be minimal in sea water but can be larger if the escape occurs during outmigration of wild salmon or trout. Ecological impacts are dependent on numbers and body size. Hence, the ecological impact will be largest if immature fish enter directly into rivers after escape and if they have reached a large body size before escape. In that case, the strongest ecological effect may be caused by predation on juveniles of Atlantic salmon and brown trout and may also include displacement from up-river spawning grounds if escapees show aggressive or dominant behavior.

4.1.2 Hazard characterization

If a large-scale escape occurred from the experimental net pens, most escapees would likely be experimental controls whose ecological and genetic impacts would not differ from those of escaped farmed salmon. Experiments have shown that the eggs of escaped farmed females are more likely to be fertilized by wild males than by escaped farmed males. This is caused by the asymmetry in how reproductive fitness is distributed. However, escaped farmed salmon spawning among themselves is demonstrated in spawning experiments and has been indicated in many rivers where the proportional farmed genetic ancestry has identified likely mating between farmed fish (Diserud et al. 2020). Moreover, some escaped farmed salmon may enter small rivers and creeks that do not normally hold wild salmon, with the result being juvenile salmon that genetically resemble farmed salmon (Pulg et al. 2020).

Escaped farmed females are also to a larger extent than wild females involved in pair formation with brown trout, leading to a higher proportion of interspecific hybrids among the offspring of escaped farmed females than among wild females (Youngson et al. 1993). Their offspring compete with wild salmon and trout in the rivers but are sterile because of different chromosome numbers in the parental species. It is possible, however, that interspecific hybrids may leave offspring with aberrant chromosome constitution.

Another group among the escapees would be F1 VIRGIN salmon that are completely sterile. Their effect would likely resemble that of immature farmed salmon, but if large-sized they might be viewed as an ecological novelty as most escaped farmed salmon are sexually mature when they are large-sized. The **impact** of large-sized immature salmon would be **moderate** if they entered several rivers and preyed on juveniles of wild salmon or trout populations, and minimal if they did not enter freshwater or did not eat while in rivers. If many immature fish entered the same river, and the fish

preyed on a weak population, the **impact** could be **temporarily high**. However, the impact should still be considered **moderate** as it would be local and will end when the sterile fish died.

A third, and smaller group among the escapees, would be physiologically fertile F1 VIRGIN salmon (or controls) with one fertile allele in addition to one sterile allele. They would have effects that differed from those of escaped farmed salmon and those of the wild-type controls. The potential spread of a sterility allele is a hazard that is not known from previous experience. VKM assesses this to be the most serious potential impact of escapes from the experiment, as it would potentially spread the sterility allele to new generations and other wild populations and not be strongly selected against because its effect is hidden in heterozygotes (Hedrick 1983). When homozygous, the sterility allele would contribute to reduced population size as homozygote sterile individuals would survive and compete as juveniles in the river but not produce offspring. Weak wild populations would be especially vulnerable for two reasons: first, escaped heterozygote individuals would experience an increased reproductive success when competing with fewer wild salmon, and secondly, the penalty in lost reproduction in recessive homozygotes would act stronger in a weak wild population than in a strong wild population.

Consider a small number of escaped experimental salmon with one fertility and one sterility allele. If they escape after one year in the net pens, they will have reached a body weight of *ca*. 4 kg and some may approach sexual maturity. Let us assume one female and one male ascended the nearest stream and spawned there. If they are the only Atlantic salmon present, the frequency of both the fertility (p) and the sterility allele (q = 1-p) = 0.5 (or 50%). If there were an equal number of wild Atlantic salmon in the stream, q = 0.25, and if there were nine times as many wild Atlantic salmon, q = 0.05.

Established theory in population genetics (e.g., Hedrick 1983) can help analyse the fate of a sterility allele in a salmon population, because it resembles well known cases in *Drosophila* and human genetics where some alleles are known to cause preadult mortality (or complete sterility) in the homozygous state (Hedrick 1983, Zschocke et al. 2023). In the theory of recessive lethals, the relative fitness of one homozygote (WT/WT) and the heterozygote (W/-) is the same, but the other homozygote (-/-) is lethal (or as here: do not produce offspring). With loss-of-function mutations (as in *dnd*-KO), it is a common observation among organisms as diverse as *Drosophila*, yeast, and humans, that there is no phenotypic difference between homozygous dominant wildtype and heterozygous loss-of-function constellations (Zschocke et al. 2023). The heterozygote would be assigned a fitness of 1 like the dominant homozygote whereas the recessive homozygote would be assigned a fitness of 0.

In such a situation, the allele frequency q will be reduced every generation because of strong selection against the recessive homozygote, but at a faster rate when q is high than when it is low (Hedrick 1983). This can be seen from the equation describing the allele frequency in generation t+1, given the allele frequency in generation t (Hedrick 1983: pp. 124-131):

$q_{t+1} = q_t /(1+q_t)$

which can be solved with respect to how many generations t it takes to change the allele frequency from its initial frequency q_0 to the allele frequency q_t in generation t:

$t = (1/q_t) – (1/q_0)$

From this equation, it can be shown that it takes only two generations to halve the allele frequency of q from 0.5 (i.e., 50% to 25%), but it takes ten generations to halve it from 0.1 and 100 generations to

halve it from 0.01. This is the reason why, for example, many single-gene diseases in humans leading to high prepubertal mortality, may persist in human populations even though being strongly selected against (Zschocke et al. 2023).

The above calculations demonstrate that when a sterility allele enters a wild Atlantic salmon population, it may persist for many generations. This also implies that there is an increased possibility that sterility alleles spread to other wild Atlantic salmon populations. VKM considers this a **massive impact** on wild Atlantic salmon populations.

The above calculation is a simplification. If an escapee carrying a sterility allele enters a dense Atlantic salmon population, it will be challenged by competition with wild spawners (Fleming et al. 1996, 1997) and the offspring must compete with wild juveniles. In this region of Norway, however, many wild Atlantic salmon are in poor status and the success of a sterile individual may be higher than in a wild population with good status. The finding of highly introgressed Atlantic salmon juveniles in small streams previously considered to be trout streams (Pulg et al. 2020) demonstrates that localities exist that could also multiply a sterility allele. The offspring of farmed (or experimental) salmon in trout streams may be highly competitive if food is abundant but may also suffer higher mortality than wild salmon offspring in the presence of trout predators (Solberg et al. 2020).

At any rate, even a single heterozygote escapee may enter the sterility allele into a wild Atlantic salmon population at higher frequencies than would be expected from natural mutation rates. In benign environmental conditions, a salmon population has the potential to increase its numbers considerably (Hansen et al. 1996). A worst-case scenario may multiply the number of heterozygous sterile fish from 1 female (and one male) in one generation to 5-10 in the next. This would also make the spread of the sterility allele to other populations more likely.

In the open ocean, the impact of the F1 VIRGIN salmon and WT controls would be **minimal** based on the current knowledge of Atlantic salmon in the ocean. Escaped farmed salmon are known to eat the same food items as wild Atlantic salmon. The survival of Atlantic salmon at sea is largely independent of the number of salmon entering the sea (Jonsson et al. 1998). In the ocean, salmon are much less numerous than many species of similar-sized marine fishes (Utne et al. 2021). At any rate, the total number of fish in the experiment is small on the geographical scale that would be relevant for ocean migrations.

4.1.3 Exposure characterization (Likelihood)

VKM assesses that the double-net construction of the experimental sea cages in this experiment may not change the risk of escape relative to conventional sea cages. The likelihood of escape in conventional aquaculture is low, even though there is considerable under-reporting in the official statistics (Skilbrei et al. 2015).

A negative trend in escaped farmed salmon in rivers has been demonstrated in River Etne when studying the numbers of escaped farmed salmon entering a fish trap during 2014-2018 (Madhun et al. 2023). At a national scale, the proportion of escaped farmed salmon in autumn samples has shown a decreasing trend from 2006 to 2022 (Wennevik et al. 2023). Yet, only three out of 13 production zones covering the entire Norwegian coast were assessed as having a low risk of continuing genetic impact caused by escaped farmed salmon, whereas six production zones, including PO4 where Matre is situated, were assessed as having a high risk of continuing genetic

impact (Solberg et al. 2023). This comes on top of high introgression already being documented in several salmon populations in western Norway (Diserud et al. 2020, 2022).

Under normal weather conditions, an escape from the net pens in Smørdalen is assessed as **very unlikely**. To our knowledge, no major escape events have been registered from the IMR Matre fish farms. Under extreme weather conditions, however, this region has experienced major escape events. There were large-scale escapes in Fensfjorden (with 51 700 salmon from one fish farm) in January 2015 when the storm Nina hit Vestland close to where Masfjorden empties into this larger fjord, and at Osterøy (with 69 300 rainbow trout from one fish farm) just outside the nearest National Salmon Fjord.

Two hazards for wild Atlantic salmon that might be caused by the research trials at Matre have been identified as different from those caused by conventional farmed salmon.

The first hazard is that some of the 303 VIRGIN salmon are capable of reproducing and transferring genetic sterility to wild salmon. Given the limited knowledge about F1 VIRGIN salmon for this experiment, we cannot exclude that some VIRGIN salmon are functionally fertile and carry a sterility allele. The likelihood of this occurring cannot be estimated with any degree of certainty because the necessary analyses have not been carried out.

In order for this major hazard to occur, the likelihood that some of the 303 VIRGIN salmon are fertile must be multiplied by the likelihood of those fish escape from captivity and enter a river where they can spawn. Even if this is **very unlikely**, the likelihood is higher than zero.

The other hazard is that VIRGIN salmon that are sterile escape at a body size and time of the year that make them potent predators on juvenile salmonids in rivers and/or on outmigrating post-smolts in fjords shortly after leaving the river. This must be considered **very unlikely** but as most rivers in the region are characterized as having poor population status for both Atlantic salmon and sea trout (VRL 2021, 2022), even limited numbers of fish escaping may make up non-negligible proportions in local rivers. A reduction of the local populations by predation may also lead to indirect genetic effects (Bradbury et al. 2020b).

For other hazards discussed, this experiment with 303 VIRGIN and 485 control farmed salmon may contribute to effects that are already documented for escaped conventionally farmed salmon. We do not, however, expect those hazards to be different from a similar number of escaped farmed salmon originating from commercial aquaculture.

4.1.4 Risk characterization

One hazard with potentially **massive impact** and one hazard with potentially **moderate impact** are identified in this assessment. Both must be considered to occur with **very low likelihood**.

It is not straightforward to quantify the risk category composed of an unlikely event that could potentially cause a massive impact. The best way to go beyond speculation is to carry out the necessary experiments under conditions of full physical containment.

Based on expert judgement, we characterize the risk caused by physiologically fertile individuals among F1 VIRGIN salmon to represent a **potentially high risk**, as fertile fish carrying a sterility allele

could introduce the sterility allele to wild populations by vertical gene transfer and spread sterility alleles to other populations and to the following generations.

We characterize the risk caused by completely sterile F1 VIRGIN salmon to be **low**. They would have **minimal impact** in the ocean, and **minor to moderate impact** in rivers, and the impacts discussed would occur with **low likelihood**. We have identified the potential predation by large-sized immature individuals on juvenile salmonids as a potential ecological novelty. Very little is known about this potential impact and experiments that could inform the assessment have not been carried out.

4.2 Impacts of GM fish on biotic components and processes

4.2.1 Hazard identification

Salmonids in rivers exert top-down effects on ecosystem structure and processes (Power 1990). It has been demonstrated that juvenile salmon with added growth hormone (and increased growth potential) may lead to a different effect on stream ecosystem function than non-manipulated juveniles (Cucherousset et al. 2021).

Among the hazards considered here, it is likely that if large immature VIRGIN salmon prey on juvenile salmonids in rivers, the food web could be altered with effects on lower trophic levels (e.g., predatory insects, algae- and litter-feeding insects, primary producers, and decomposers) during the years when this occurred but limited in time to the maximum age of immature VIRGIN salmon.

Offspring of fertile heterozygotes (carrying one fertile allele and one sterile allele) could also affect biotic components and processes through top-down effects, but we do not consider this to be different from offspring of escaped farmed salmon.

4.2.2 Hazard characterization

If VIRGIN salmon of considerable body size enter rivers and start feeding on juvenile salmon or trout, it would constitute an ecological novelty. The river ecosystem could be changed as a top predator was added that would not fall victim to mergansers or herons. This could reduce the number of juvenile salmonids in the stream and thereby affect the number of individuals and/or productivity at lower levels in the food web (cf. Cucherousset et al. 2021). We consider the impact of immature salmon on biotic components and processes in the river to be **moderate**.

4.2.3 Exposure characterization (Likelihood)

The likelihood that large immature VIRGIN salmon escape and start to prey on juvenile salmonids in rivers or during their post-smolt migration in the fjords is considered **very unlikely** based on current knowledge. A similarly low likelihood must be assigned to effects on other biotic components and processes.

4.2.4 Risk characterization

If a predation pressure from large immature VIRGIN salmon on small fish is rivers is realized, it would be considered a **moderate impact** occurring with a **very low likelihood**. Because of the limited extent in time and space, we consider the overall risk to be **low**. This is assessed with **high uncertainty**.

4.3 Fish pathogens, infections, and diseases

4.3.1 Hazard identification

A number of micro- and macro parasites infect wild and farmed salmon. The outcome of infection is determined by a complex relationship between factors pertaining to the host, the infectious agents (there may be more than one) and the environment they interact within (Snieszko, 1974).

The hazards identified in relation to pathogens, infections and diseases are (Figure 10):

1) That F1 VIRGIN salmon get infected and spread infectious agents when held in confined aquaculture (open net pens) at Matre.

2) That the F1 VIRGIN salmon get infected and spread infectious agents after escape from confined aquaculture at Matre.

In line with the comparative approach of the ERA, the question at hand is whether the genetic modifications in F1 VIRGIN salmon have unintended and/or unknown outcomes that negatively impact the general robustness, or more specifically the susceptibility to infectious agents in F1 VIRGIN salmon. Accordingly, whether the interaction of F1 VIRGIN salmon with the pathogen and environment result in a more negative outcome for the environment than the comparators would have in the same interaction. Relevant comparators are conventional farmed salmon, while the comparator in the experiment is, as previously described, the wild-type comparator (WT-siblings of F1 VIRGIN salmon). It should be noted that the WT-comparator, as siblings to the F1 VIRGIN salmon test fish, will be more like these test fish than conventional salmon are (except in the *dnd* gene).

The impact may be conveyed as a cascade effect that can be visualised as one individual (case 1) in a cage becoming infected and infectious and that this leads to transmission of pathogen to other susceptible fish in the same cage. The next step will then be to transmit the pathogen to new susceptible hosts in other cages at the same site and the next, for instance, to susceptible wild and farmed fish in the surrounding environment, neighbouring sites and so on.

The initially infected fish (Case 1) in the defined cage must also have been exposed to the pathogen by sharing environment or water with a farmed or wild source of infection. A cascade effect thus requires a source and that susceptible hosts are available and become infectious such that the basic reproduction ratio exceeds the transmission threshold, $R_0 = 1$.

With the comparative approach, the question is whether F1 VIRGIN-salmon has characteristics that make it a more susceptible Case 1, or in other ways can have a positive impact on the reproduction number R. Besides the basic question regarding host susceptibility, the factor that determines the size of R and that could be changed by altered host characteristics is the period of time over which the infected host remains infectious. R will increase with the length of the period before immunity or death of the infected host.

4.3.2 Hazard characterization

More than 435 million farmed Atlantic salmon are primarily held in open net pens along the coast of Norway (Directorate of Fisheries). Open design of aquaculture systems in the sea allows close interaction between farmed fish contained within the nets and the environment, including wild and contained farmed fish in the vicinity.

Infectious agents, including the ectoparasitic salmon louse severely challenge the aquaculture industry by causing disease, mortality, degradation, and lost production. The environmental impact of salmon louse propagated and released from aquaculture is well documented and includes mortality in outmigrating wild salmon which leads to reduced return of spawning salmon and harvestable surplus in the most aquaculture-intensive areas in Norway (VRL 2020, Vollset et al. 2016, Shephard & Gargan 2021, Vollset et al. 2023). Currently, estimates of mortality in outmigrating wild post-smolt due to salmon louse is the sole indicator in the system that regulates expansion of the aquaculture industry, the so-called traffic light system (FOR-2023-02-28-270 Produksjonsområdeforskriften).

The infection status of farmed salmonids is under constant scrutiny by fish health services, the Food safety authority, laboratories and research institutes, and there is a high degree of transparency and data sharing between the industry, authorities and the scientific community. Accordingly, the health status and health challenges of farmed fish are well known and documented in technical and scientific literature, including the annual Fish health reports (Sommerset et al. 2023). The high number of outbreaks of a variety of parasitic, bacterial and viral diseases affecting a large proportion of the farmed biomass result in release of infectious agents into the environment and exposure of other farmed salmon as well as wild salmon. The environmental impact of these infections is however less studied than the impact of salmon lice (Raynard et al. 2007, Johansen et al. 2011, Grefsrud et al. 2023, VRL 2023).

When infected farmed salmon escape, they can enter fjords, estuaries, rivers and lakes that are otherwise unavailable for either salmon in farms or seawater currents that are carrying infectious agents. Some of the infectious agents that cause disease in the farming industry have a higher incidence in escaped farmed salmon than in wild salmon (Garseth et al. 2013, Madhun et al. 2015). It is considered likely that escape of infected farmed salmon contributes to spread of infections to wild fish. For instance, farmed escapees contributed to spread of the bacterium causing typical furunculosis from infected fish farms to wild salmonids with the import of salmon smolt in 1985 that were infected with *Aeromonas salmonicida* subsp. *salmonicida* (Johnsen & Jensen 1994).

Site 12154 Smørdalen where the F1 VIRGIN salmon potentially will be studied, is in Production area 4 (PO4), which extends from Nordhordland to Stadt. Both Atlantic salmon and rainbow trout are produced in PO4. PO4 has been coloured red according to the traffic light system, i.e., the mortality in outmigrating wild smolt is estimated to be more than 30% due to salmon lice infestation. The mortality rate in farmed salmon in PO4 in 2022 was 22% and thus higher than the total average for Norway which was 16.1% (Sommerset et al. 2023). In PO4, rainbow trout had a mortality rate of 14.5%, while total average values for Norway were 17.1%. Infectious diseases and the use of non-medicinal treatment of salmon lice infestation are considered the main causes of mortality within aquaculture in general (Sommerset et al. 2023). In PO4, there are several ongoing endemics in farmed fish, including the notifiable viral diseases infectious salmon anaemia (ISA), pancreas disease

(PD) caused by salmonid alphavirus 3 (SAV 3) and the non-listed viral diseases heart and skeletal muscle inflammation (HSMI) caused by piscine orthoreovirus –1 (PRV-1) and cardiomyopathy syndrome (CMS) caused by piscine myocarditis virus (PMCV). PO4 also has several cases of bacterial disease outbreaks including pasteurellosis (*Pasteurella* sp.) and winter ulcers (*Moritella viscosa* and *Tenacibaculum* sp.). Accordingly, the health status of farmed salmon in PO4 is worrisome.

According to the applicant, the only vaccine that will be used before sea transfer is Clynav (Elanco). The indication for this vaccine is active immunization to reduce clinical symptoms and pathology associated with pancreas disease caused by salmonid alphavirus 3 (SAV 3) (Felleskatalogen 2020). It is also quite common to vaccinate farmed salmon to immunize against a number of bacteria, for instance *Aeromonas salmonicida* subspecies *salmonicida*, *Vibrio anguillarum*, *Vibrio salmonicida* and *Yersinia ruckeri.* However, these vaccines are not planned for this experiment.

The outcome of infection at the individual and population level in wild fish has not been studied for all infections mentioned above. In general, infections can result in a variety of outcomes from rapid immunization and recovery, development of covertly infected fish (healthy carriers), reduced fitness, development of disease and mortality, including predation. Depending on the characteristics of the infectious agent and the host, some infectious agents will spread further and self-sustain within wild populations. Other less transmissible or more virulent infections may die out or remain present at a low level. *Aeromonas salmonicida* ssp. *salmonicida* is an example of an introduced infectious agent that was spread from farmed salmon to wild populations and that still, nearly 40 years after the introduction, has an impact both on wild and farmed populations. In farmed populations, most salmon transferred to the sea are vaccinated, leading to vaccine-related side effects. Additionally, in wild salmonids, occasional outbreaks of typical furunculosis result in high mortality in some rivers draining to the Namsen Fjord (Garseth et al. 2022). Due to the low number of F1 VIRGIN salmon in the experiment, the magnitude of impact is assessed to be **minor**, i.e., potential impact on local populations.

4.3.3 Exposure characterization (Likelihood)

Assessment of likelihood of the identified hazards in relation to pathogens, infections and diseases are (Figure 10):

1) That F1 VIRGIN salmon get infected and spread infectious agents when held in confined aquaculture (open net pens) at Matre.

2) That the F1 VIRGIN salmon get infected and spread infectious agents after escape from confined aquaculture at Matre.

Factors affecting the likelihood of undesirable events caused by infections in F1 VIRGIN salmon are in general their health and immune status, the number of F1 VIRGIN salmon, and the duration of the release of pathogens which depends on how early disease is detected and what actions are taken if/when disease is detected.

According to the application, the study will be terminated at detection of disease. If F1 VIRGIN-salmon develop disease, they have been infected by a pathogen that is present in a farmed or wild reservoir that is already present in the environment. It is very likely that F1 VIRGIN salmon and WT-comparators are infected with infectious agents present in their current freshwater environment, and that they will become infected with new infectious agents that are present in the sea water environment at Matre. Inclusion and exclusion criteria for initiating the endpoint have not been

defined in the application and there is no differentiation between groups of diseases. For instance, a differentiation could be considered between listed vs. non-listed infectious diseases. For listed diseases, a defined pathogen is a necessary, but not always a sufficient cause of disease. In addition, the reservoir of the pathogen is often well defined. In multifactorial diseases, the main component or cause of disease is not necessarily is a specific pathogen. Early detection of disease and subsequent termination of the study will reduce the likelihood of a harmful event occurring.

The health status of F1 VIRGIN salmon at the time of transfer to sea is under the scrutiny of the fish health service. According to regulations, the fish health should be checked within three weeks prior to transfer to sea. It is therefore unlikely that F1 VIRGIN salmon are infected with infectious agents that cause serious listed diseases at the time of transfer to Smørdalen. Early detection of infectious diseases is necessary to prevent spread of the infectious agent to other fish and units at the facility. Close monitoring increases the likelihood of detecting disease and depends on the vigilance and competence of those who farm the fish daily.

Since VKM uses a comparative approach in the ERA, the first question is whether F1 VIRGIN salmon are more susceptible to infections or are less robust than the comparators. There are no data in the application that makes it possible to assess this. There are no records of infectious agents that the test and control groups have been exposed to in the freshwater environment. Furthermore, there are no comparative registrations of cause specific mortality, results from relevant analyses or investigations that could elucidate potential differences in susceptibility between groups. The reports from the fish health service (veterinary reports) state that the health of GMO fish (F1 VIRGIN salmon and the WT comparator) is “ok”, meaning that there are no findings that are notifiable to the animal health authorities or that requires action. The conclusions from these reports are thus not based on comparing the response in two groups after a known or unknown similar exposure.

Evaluation of operational welfare indicators (OWI) could shed light on possible differences in robustness, including how prone the groups are to develop lesions in the skin or gills that could act as entry points for infectious agents. OWI are however outcomes of various exposures in the environment and not specific enough with regards to predicting susceptibility to specific infections. Comparative measurements of for instance skin thickness or mucus quality including content of immune active components have not been performed.

The F1 VIRGIN salmon and their WT siblings are offspring of two male and four female brood fish. Other than that, the application does not describe the exact pedigree of the F1 VIRGIN salmon and wildtype salmon among the possible combinations of males and females of these brood fish. Nevertheless, there is reason to believe that there is a narrow genetic diversity including immune diversity (MHC alleles) in both the F1 VIRGIN group and the WT comparator group compared to conventional farmed salmon and wild salmon.

VKM concludes that the likelihood of F1 VIRGIN salmon being more susceptible to infection than its experimental comparator (WT siblings) is unknown. The limited number of parents (two males by four females parenting both groups) introduces risk of confounding of e.g., genetic effects of male parent and effects of introduced mutation (sterility) (cf. chapter 2.3.1). Based on an expert opinion, the F1 VIRGIN-salmon are **unlikely** to be more susceptible to infection than the comparators, i.e., negative consequences would be expected to occur with a likelihood of 5-10%.

The second step to assess in the chain of events leading to harm is whether a potentially higher susceptibility in F1 VIRGIN salmon result in release of pathogens to the environment, and with the comparative approach, if the magnitude of release of infectious agents is higher than in the Comparators. Based on expert opinion, the likelihood is **very unlikely**, i.e., negative consequences would be expected to occur with a likelihood of 0-5%.

The third step to assess is whether potential increased release of infectious agents from F1 VIRGIN – salmon to the environment will have a stronger environmental impact than release from the comparators. Based on expert opinion, the likelihood is **very unlikely**, i.e., negative consequences would be expected to occur with a likelihood of 0-5%.

The overall conclusion from VKM is that the likelihood that more harm to the environment will be caused by spread of infection from 303 F1 VIRGIN salmon in confinement at Matre than from comparators is **very unlikely**, i.e., negative consequences would be expected to occur with a likelihood of 0-5%

The second hazard, that the F1 VIRGIN salmon spread infectious agents after escape from confinement at Matre depends on the likelihood of escape as presented earlier in the report. The likelihood of an environmental impact also depends on the host-pathogen interaction, how many infected F1 VIRGIN salmon that survive and how they are distributed in space and time in the environment. The likelihood and magnitude of harm are higher if several F1 VIRGIN salmon enter one river and interact with local salmonids simultaneously than if they are spread over a large area and only a few enter each river.

The overall conclusion from VKM is that the likelihood that more harm to the environment will be caused by spread of infection from escaped F1 VIRGIN salmon than from escaped comparators is **very unlikely**, i.e., negative consequences would be expected to occur with a likelihood of 0-5%.

4.3.4 Risk characterization

Both the magnitude of impact and the likelihood of impact are affected by the number of F1 VIRGIN salmon and wild-type siblings in the experiment.

To be able to disentangle the hazards identified concerning fish pathogens, infections and diseases, there is a need for specific information about the infection and health status of F1 VIRGIN salmon compared with the same status of comparators (wild type comparator i.e., siblings of F1 VIRGIN salmon, and conventional farmed salmon). Unfortunately, and as previously described, neither a targeted research design nor laboratory methods have been applied to investigate possible differences in immunocompetence and susceptibility to infectious agents between VIRGIN salmon and the comparator.

VKM has requested clarification regarding how the project will monitor and assess the health in the test group (VIRGIN salmon) and the comparator after sea transfer. The applicant replies that the project will follow the normal health control program at the station, with veterinary inspection regularly. On request from VKM, the applicant has also provided reports from routine health controls of the 303 F1 VIRGIN salmon and comparator that have been performed by an independent commercial fish health service (Aquavet Gulen) at Matre.

It should be noted that the primary objective of monthly routine fish health checks conducted by health services is to detect impaired fish welfare and infectious diseases, in particular notifiable diseases, to avoid spread of selected infectious agents by transfer of fish to new locations. Routine health checks are thus not designed, nor conducted in a manner that provides detailed information about contrasting health status between groups in research, for instance, as requested here,

between F1 VIRGIN salmon and the WT-sibling comparators. Accordingly, one would expect that the applicant commissioned the fish health service or other skilled staff to conduct a more targeted surveillance program that could inform the applicant and the ERA about potential contrasting health status between F1 VIRGIN salmon and the comparator.

With the regular routine fish health checks, the only information available is that infectious diseases have not been detected in F1 VIRGIN salmon and apparently neither in the wildtype comparator group. There is therefore also a lack of information about what the F1 VIRGIN salmon and WT-comparator group have been exposed to in the research facility, if any.

According to the application, one of the main advantages of sterile VIRGIN salmon is the lack of germ cells, and that by using germ cell free salmon one prevents puberty and thus avoids significant welfare issues such as reduced growth, lower fillet quality, and increased susceptibility to diseases. Besides this description, the application does not provide documentation of reduced susceptibility to infectious diseases in F1 VIRGIN salmon compared to conventional farmed salmon.

It should also be noted, that although puberty and sexual maturation may contribute to development of clinical disease in salmon, it is not a necessary component. Sexual maturation also occurs relatively late in the production cycle in conventional farmed salmon.

In conclusion, neither a targeted research design nor laboratory methods have thus far been applied to investigate possible differences in immunocompetence and susceptibility to infectious agents between VIRGIN salmon, the WT-comparator, and conventional farmed salmon. Furthermore, the application does not specify activities that will address or close this knowledge gap in the experiment in question. On the contrary, the applicant states that the study will be terminated if a disease outbreak occurs. Overall, the project lacks a targeted process or pipeline for generating knowledge and comparable data on this subject. Data provided by the applicant is not sufficient to evaluate the presence of unintended or unknown outcomes of the gene modification that could have an impact on the host-pathogen-environment interaction, for instance the ability of F1 VIRGIN salmon to mount an immune response towards infection with specific pathogens. Thus far, the studies have focused on growth and welfare traits that are not necessarily indicators of how test animals interact with infectious agents.

The low number of F1 VIRGIN salmon and WT comparators included in the study limit the ability of the study to detect rare outcomes of the gene modification. On the other hand, the low number of VIRGIN salmon included also means that an unknown and unintended effect must have a high impact or effect on the individual level to have a negative impact on the environment.

Based on a comparative approach, the environmental **risk** with regards to spread of infectious agents from release of 303 F1 VIRGIN salmon is assessed to be **low**, both if F1 VIRGIN salmon are contained at Matre, and if F1 VIRGIN salmon escape.

VKM assess the level **uncertainty** to be **high** in both assessments since available information on the topic is limited, and mostly expert judgements have been used.

4.4 Impacts of GM fish on human health

The Terms of Reference for the risk assessment states that impacts of GM fish on human health are to be considered other than from food (intake of GMO fish). Handling of the VIRGIN salmon, where the only trait is sterility, would not imply human exposure that is different from the handling of other farmed fish and therefore not part of the assessment.

5 Risk-reducing measures

VKM assesses that the following risk-reducing measures could have been taken by the applicant.

- Whereas the production of rescued F0 individuals, followed by generation of F1 individuals based on these, is an acceptable strategy for production of the limited number of fish to be included in the applied experiment, this strategy is not feasible on a larger scale. This is because of the considerable uncertainty caused by generation of multiple allelic variants and mosaics in the F0 individuals. With the chosen strategy, random and unique mutations will be generated when the procedure is repeated, meaning that new unique individuals are produced every time, i.e., results are unpredictable.
  A more conservative alternative approach to reduce the number of unique mutations would be to generate individuals based on strategies frequently used to breed research animals. I.e., starting with identification of male and female fish that are heterozygote for the same mutation in the *dnd* gene (e.g., the exact same 8-der). Oocytes are then fertilized and co-injected with the rescue RNA. Such a cross can be expected to produce around 25 % fertile fish that are homozygous for the *dnd* gene (but not sterile due to rescue). Detailed molecular characterization can then be performed on individuals with this unique modification. Risks caused by mosaicism in the F0 founders, involving screening for multiple alternative alleles, will then be removed. For this, a reliable method can also be developed to detect a single unique genetic modification.

- Confirmative genotyping of fin-clips from the chosen 303 experimental and 485 control fish would have reduced the potential of errors from handling and characterizing the 2200 samples that were used to select them.

- An external marker on all PIT-tagged individuals would increase the likelihood that they are treated as GM-fish when recaptured by others than those equipped with PIT readers/antennas.

- A broader vaccine approach could provide a better protection of released fish and could also be used to harvest experimental data by measuring the response in test and control groups.

- Several experiments could be carried out that would have informed this risk assessment. Facilities exist that would allow the experiments to take place in full containment.

6 Uncertainties and data gaps

6.1 Uncertainties

Uncertainties and data gaps

The quantification of hazards for this application has not been attempted by the applicant.

VKM has identified uncertainties in the **molecular characteristics** of the experimental fish, both in the group of 303 F1 VIRGIN salmon and in their sibling controls:

- Correspondence between the *dnd* knockout genotype in adipose fin-clips and the germ cell-free phenotype in F0 rescued salmon has been shown in a limited number of individuals and discrepancy has been reported due to mosaicism.

- Information on a confirmative second genotyping of all individuals included in the study, both wildtype (485) and fish with double knockout alleles (303), is missing. Undeliberate errors may have been introduced in the analysis of 2200 samples due to sample quality, sample handling, selection and marking of the individuals to be included in the study. Confirming the data is essential for the determination of the genetical background included in the study.

- Limited information is available about the less common mutations included in the study, since the presented data confirming sterility only covers the most common mutations. Of the F1 VIRGIN material, 45% have a knockout genotype (ins1/del8 and del8/del8), which is previously shown to produce germ cell-free individuals in F1 VIRGIN fish (shown among 7 siblings and 8 of 28 fish from an earlier crossing). Of the remaining 303 VIRGIN fish, 48% have *dnd* mutations reported to cause sterility in F0 germ cell free CRISPR/Cas9 edited fish with somewhat lower correspondence with sterility. The final 7% of the *dnd* mutations represented in the 303 VIRGIN fish are less well-characterised genotypes, where lack of gonad development has not been confirmed by dissection, i.e., morphological, or histological examination. The confirmatory analyses of the mutations present in the 303 F1 VIRGIN fish produced for this experiment by crosses in November 2021 included only dissection and histology of seven knockout siblings (all ins1/del8) and two wildtype individuals. Of these, samples from two specimens were lost during histology preparation.

- The control population cannot be said to represent conventional farmed salmon as they are based on only a very limited number of parents. Genetic confounding increases when only two males by four females are used to produce the 303 F1 VIRGIN salmon and their 485 wildtype sibling controls. In addition, information about family representation among the two males by four females crossing in the different experimental groups is missing. This may introduce biased estimates of effects and hence reduce the validity of the results.

VKM has identified uncertainties in the **environmental risk assessment**:

- The experimental environment with four small 5 x 5 m net pens, 5 m deep, located within a larger net pen, although like commercial net pens with respect to holding the same water as the fjord they are in, is unlikely to mimic the environmental conditions and growth opportunities in net pens that are 50 m in diameter and up to 50 m deep.

- The behaviour of VIRGIN salmon, and that of their offspring if they are not fertile, has not been studied.

- The experimental design does not follow EFSA (2013) guidelines on choice of comparators in situations where escape from captivity is possible.

- The lack of external marks on the experimental fish (PIT-tagged F1 VIRGIN salmon and wildtype siblings) adds uncertainty as to where experimental fish might spread if escaping from the facility at Matre.

6.2 Data gaps

Proof of sterility in all of the given genotypes of the 303 VIRGIN F1 salmon is lacking; only some of the common genotypes are confirmed sterile and information on confirmational genotyping to avoid undeliberate errors in sample handling is missing.

Behaviour of VIRGIN salmon in freshwater environments with gravel substrate and running water is not known. This relates to competition and to potential predatory behaviour as well as potential spawning behaviour.

No plan on how to study the response of experimental fish to infectious agents, that are likely to be introduced to the research material within the planned 1.5 years in net pens, has been presented.

7 Conclusions

VKM assesses that the research trial on releasing VIRGIN salmon into sea cages from autumn 2023 to February 2025 is associated with **potentially high risk** for wild Atlantic salmon. This is mainly based on two concerning elements; First and foremost, based on the information provided in the application, VKM concludes that there is insufficient documentation on proof of sterility in all the 303 F1 VIRGIN individuals and a possibility that an unknown number of potentially fertile individuals of F1 VIRGIN salmon with allelic mutation(s) in the *dnd* gene are erroneously registered as double allelic mutants (n = 303). Likewise, an unknown number of the homozygous wildtype controls (n = 485) may in fact be fertile heterozygous fish, carrying a mutated *dnd* allele. Secondly, VKM assesses that it is uncertain whether the likelihood of fish escaping from the experimental pens differs from the likelihood of escaping from standard net pens used in fish farming.

It is therefore possible that experimental fish, should they escape, can spawn with wild salmon, and if carrying sterility alleles, can introduce sterility alleles to wild Atlantic salmon populations by vertical gene transfer. The sterility allele will be recessive and therefore hidden to purging in wild populations, except when in the homozygote state in the second and later generations. Sterile recessive homozygotes will compete for food and space as juveniles in rivers, and thereby reduce the productivity of wild populations and reduce the viability of vulnerable populations.

The status of wild Atlantic salmon populations is poor in the region (Vestland county) where Matre is situated. The typically wide dispersal of escaped farmed salmon and the fact that the experimental fish bear no external mark telling them apart from ordinary farmed escapees, imply that salmon populations can be (unknowingly) affected even when located far away from Matre.

VKM assesses that spread of sterility alleles to future generations of wild salmon populations would be a **massive impact** on wild Atlantic salmon. The overall likelihood of such spread, including salmon escaping from the experiment, is assessed as **very unlikely**. However, sterility alleles can be introduced to wild populations by very few individuals. VKM assesses that a massive impact occurring with very low likelihood leads to the conclusion that the experiment poses a **potentially high risk** to wild salmon.

If sterile, large-sized fish escape into rivers they could act as an ecological novelty if preying on juvenile salmon and trout. It is not uncommon for escaped (conventional) farmed salmon to enter rivers as immature fish, but they are typically smaller than escaped farmed salmon that have reached sexual maturity. VKM assesses the risk posed by sterile large-sized salmon in relation to predation on juvenile salmonids in rivers, and other ecological interactions like competition for food, to be **low**. This is assessed with **high uncertainty**, as relevant experiments have not been carried out.

Impacts on biotic components and processes, mediated by top-down ecological effects in river food webs by large-sized sterile salmon feeding on juvenile salmonids, are characterized by VKM as a moderate impact occurring with very low probability. The associated risk is considered **low**.

The risk to wild salmon populations with regards to spread of infectious agents from release of 303 F1 VIRGIN salmon is considered **low** (low likelihood and small impact), both if F1 VIRGIN salmon are contained at Matre, and if F1 VIRGIN salmon escape.

In general, there is **high uncertainty** related to VKM's assessments based on the data received from the applicant. Several of the knowledge gaps identified by VKM can be filled by performing studies in contained facilities. This would reduce uncertainties related to the risk assessment but would not necessarily reduce the likelihood of unwanted effects.

A thorough molecular characterization of the Atlantic salmon intended for release into sea cages is needed to fully assess the potential risks to the environment in case of accidental escape. This characterization should include confirmational genotype analysis of all individuals included in the experiment, and confirmation that each unique mutated *dnd* allele present among the experimental fish leads to loss-of-function.

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9 Appendix 1

9.1 The 303 F1 VIRGIN salmon with double allelic *Dnd* mutation

#	Fish bar code	Dnd mutation type	#	Fish bar code	Dnd mutation type
1	P10-F9	del10/del10	45	P2-H8	del7/del5
2	P13-F10	del10/del10	46	P6-H12	del7/del5
3	P16-C8	del10/del10	47	P8-A6	del7/del5
4	P17-C8	del10/del10	48	P9-D6	del7/del5
5	P19-E10	del10/del10	49	P9-E6	del7/del5
6	P20-B1	del10/del10	50	P9-H9	del7/del5
7	P21-A9	del10/del10	51	P10-D7	del7/del7
8	P21-B7	del10/del10	52	P10-F12	del7/del7
9	P22-F1	del10/del10	53	P10-H9	del7/del7
10	P3-B1	del10/del10	54	P12-G3	del7/del7
11	P3-C9	del10/del10	55	P2-A1	del7/del7
12	P5-D10	del10/del10	56	P3-E2	del7/del7
13	P7-H6	del10/del10	57	P4-C9	del7/del7
14	P10-C9	del10/del5	58	P7-G12	del7/del7
15	P11-H5	del10/del5	59	P7-G9	del7/del7
16	P12-G1	del10/del5	60	P8-A2	del7/del7
17	P15-A12	del10/del5	61	P8-H11	del7/del7
18	P17-G6	del10/del5	62	P9-G4	del7/del7
19	P20-H7	del10/del5	63	P4-B6	del7/Ins1
20	P21-A1	del10/del5	64	P12-E12	del8/del10
21	P3-C3	del10/del5	65	P12-F7	del8/del10
22	P5-F11	del10/del5	66	P13-G12	del8/del10
23	P23-A3	del2/del2	67	P13-G2	del8/del10
24	P5-B11	del2/del2	68	P14-G8	del8/del10
25	P9-C6	del2/Ins5	69	P16-A11	del8/del10
26	P17-F8	del28/del28	70	P16-H6	del8/del10
27	P10-B4	del28/Ins5	71	P18-G6	del8/del10
28	P3-H6	del4/del4	72	P19-B1	del8/del10
29	P10-A3	del7/del10	73	P19-D3	del8/del10
30	P16-A9	del7/del10	74	P1-C9	del8/del10
31	P4-A6	del7/del10	75	P20-D1	del8/del10
32	P4-H2	del7/del10	76	P20-G6	del8/del10
33	P5-D5	del7/del10	77	P21-A6	del8/del10
34	P19-C8	del7/del2	78	P22-G1	del8/del10
35	P4-A11	del7/del28	79	P2-F12	del8/del10
36	P10-A7	del7/del5	80	P3-A5	del8/del10
37	P10-F4	del7/del5	81	P3-F10	del8/del10
38	P11-B6	del7/del5	82	P6-B2	del8/del10
39	P11-E4	del7/del5	83	P6-C1	del8/del10
40	P11-F2	del7/del5	84	P7-B1	del8/del10
41	P11-H7	del7/del5	85	P9-A4	del8/del10
42	P17-E4	del7/del5	86	P2-D1	del8/del2
43	P19-C1	del7/del5	87	P4-G11	del8/del2
44	P2-G10	del7/del5	88	P14-H10	del8/del28

89	P17-G11	del8/del28	134	P22-G9	del8/del7
90	P1-B3	del8/del28	135	P2-H4	del8/del7
91	P20-E1	del8/del28	136	P4-G10	del8/del7
92	P4-G5	del8/del28	137	P6-A1	del8/del7
93	P5-D12	del8/del28	138	P6-A5	del8/del7
94	P12-F1	del8/del4	139	P6-D3	del8/del7
95	P5-G7	del8/del4	140	P6-G11	del8/del7
96	P10-G6	del8/del5	141	P6-G2	del8/del7
97	P11-C10	del8/del5	142	P8-A7	del8/del7
98	P11-C3	del8/del5	143	P8-D9	del8/del7
99	P17-F6	del8/del5	144	P9-E11	del8/del7
100	P19-D6	del8/del5	145	P9-H5	del8/del7
101	P1-C7	del8/del5	146	P10-B1	del8/del8
102	P20-E4	del8/del5	147	P10-B10	del8/del8
103	P20-E7	del8/del5	148	P10-B11	del8/del8
104	P21-F1	del8/del5	149	P10-C2	del8/del8
105	P22-C11	del8/del5	150	P10-D6	del8/del8
106	P22-F9	del8/del5	151	P10-D8	del8/del8
107	P22-H2	del8/del5	152	P10-E11	del8/del8
108	P2-G4	del8/del5	153	P10-E12	del8/del8
109	P5-D6	del8/del5	154	P10-G11	del8/del8
110	P7-E12	del8/del5	155	P10-H11	del8/del8
111	P7-F1	del8/del5	156	P11-A8	del8/del8
112	P7-G10	del8/del5	157	P11-A9	del8/del8
113	P8-E3	del8/del5	158	P11-C2	del8/del8
114	P9-E3	del8/del5	159	P11-D10	del8/del8
115	P10-B6	del8/del7	160	P11-E1	del8/del8
116	P11-C1	del8/del7	161	P12-A4	del8/del8
117	P12-F4	del8/del7	162	P12-B7	del8/del8
118	P12-H2	del8/del7	163	P12-C7	del8/del8
119	P14-E12	del8/del7	164	P12-C8	del8/del8
120	P14-F7	del8/del7	165	P12-F10	del8/del8
121	P15-F3	del8/del7	166	P13-E3	del8/del8
122	P16-C10	del8/del7	167	P13-H2	del8/del8
123	P17-D4	del8/del7	168	P16-A8	del8/del8
124	P18-B3	del8/del7	169	P16-B4	del8/del8
125	P18-E5	del8/del7	170	P16-B7	del8/del8
126	P19-C10	del8/del7	171	P16-C9	del8/del8
127	P19-E1	del8/del7	172	P16-D10	del8/del8
128	P19-G2	del8/del7	173	P16-D6	del8/del8
129	P19-H5	del8/del7	174	P16-E12	del8/del8
130	P1-A8	del8/del7	175	P16-F2	del8/del8
131	P20-F6	del8/del7	176	P16-G10	del8/del8
132	P22-C7	del8/del7	177	P16-G5	del8/del8
133	P22-G7	del8/del7	178	P16-H7	del8/del8

179	P17-A8	del8/del8	224	P22-H12	del8/del8
180	P17-E2	del8/del8	225	P23-A1	del8/del8
181	P17-E9	del8/del8	226	P23-B3	del8/del8
182	P18-A10	del8/del8	227	P23-C4	del8/del8
183	P18-B7	del8/del8	228	P23-D3	del8/del8
184	P18-C10	del8/del8	229	P23-F5	del8/del8
185	P18-D5	del8/del8	230	P23-H1	del8/del8
186	P18-F4	del8/del8	231	P2-A10	del8/del8
187	P18-F6	del8/del8	232	P2-B2	del8/del8
188	P18-H1	del8/del8	233	P2-B9	del8/del8
189	P19-A1	del8/del8	234	P2-C5	del8/del8
190	P19-D5	del8/del8	235	P2-F3	del8/del8
191	P19-E2	del8/del8	236	P2-G3	del8/del8
192	P19-F1	del8/del8	237	P2-H11	del8/del8
193	P19-F4	del8/del8	238	P3-A10	del8/del8
194	P19-F7	del8/del8	239	P3-A12	del8/del8
195	P19-H7	del8/del8	240	P3-C1	del8/del8
196	P1-A1	del8/del8	241	P3-C12	del8/del8
197	P1-A4	del8/del8	242	P3-F8	del8/del8
198	P1-D12	del8/del8	243	P3-H9	del8/del8
199	P1-E1	del8/del8	244	P4-D4	del8/del8
200	P1-F10	del8/del8	245	P4-D9	del8/del8
201	P1-F6	del8/del8	246	P4-E1	del8/del8
202	P1-G4	del8/del8	247	P4-G7	del8/del8
203	P1-H6	del8/del8	248	P5-D9	del8/del8
204	P1-H9	del8/del8	249	P5-F2	del8/del8
205	P20-C3	del8/del8	250	P5-G11	del8/del8
206	P20-C6	del8/del8	251	P5-G9	del8/del8
207	P20-D10	del8/del8	252	P6-A12	del8/del8
208	P20-D2	del8/del8	253	P6-B3	del8/del8
209	P20-E8	del8/del8	254	P6-C10	del8/del8
210	P20-G2	del8/del8	255	P6-C6	del8/del8
211	P20-H2	del8/del8	256	P6-C7	del8/del8
212	P21-E12	del8/del8	257	P6-E6	del8/del8
213	P21-E4	del8/del8	258	P6-F3	del8/del8
214	P21-E5	del8/del8	259	P6-F7	del8/del8
215	P21-F3	del8/del8	260	P6-H2	del8/del8
216	P21-G8	del8/del8	261	P7-B2	del8/del8
217	P21-H10	del8/del8	262	P7-B6	del8/del8
218	P22-A1	del8/del8	263	P7-D5	del8/del8
219	P22-B11	del8/del8	264	P7-F6	del8/del8
220	P22-D3	del8/del8	265	P7-H5	del8/del8
221	P22-D5	del8/del8	266	P7-H9	del8/del8
222	P22-E11	del8/del8	267	P8-A10	del8/del8
223	P22-E6	del8/del8	268	P8-B1	del8/del8

269	P8-B6	del8/del8
270	P8-C10	del8/del8
271	P8-C2	del8/del8
272	P8-E1	del8/del8
273	P8-E7	del8/del8
274	P8-F12	del8/del8
275	P8-G4	del8/del8
276	P8-H10	del8/del8
277	P8-H7	del8/del8
278	P9-A10	del8/del8
279	P9-A2	del8/del8
280	P9-C1	del8/del8
281	P9-C10	del8/del8
282	P9-F3	del8/del8
283	P9-F4	del8/del8
284	P10-C4	del8/ins1
285	P10-H12	del8/ins1
286	P10-H7	del8/ins1
287	P20-A3	del8/ins1
288	P21-D9	del8/Ins1
289	P23-D2	del8/Ins1
290	P3-D6	del8/ins1
291	P4-G8	del8/ins1
292	P22-E1	del8/Ins10
293	P6-E1	del8/ins10
294	P22-F4	del8/ins4
295	P13-F6	del8/Ins5
296	P21-G10	del8/Ins5
297	P14-C4	Ins1/del28
298	P3-G4	Ins1/Ins1
299	P5-E6	Ins1/Ins1
300	P8-A4	Ins1/Ins1
301	P9-H2	Ins1/ins5
302	P19-G5	Ins5/Ins10
303	P22-G11	Ins5/Ins10

Appendix 2

Timeline of application

18. april	VKM mottar søknad fra Havforskningsinstituttet (versjon 1) via Miljødirektoratet. Klokken starter.
9. mai	VKM påpekte en rekke vesentlige mangler ved søknaden og konkluderte med at dokumentasjonen ikke var tilstrekkelig til å utarbeide en risikovurdering (vedlegg 1).
11. mai	Miljødirektoratet iverksetter klokkestopp i saksbehandlingen
22. juni	VKM mottar oppdatert søknad (versjon 2 – ny informasjon i rød tekst) via Miljødirektoratet. Klokkestoppen oppheves.
30. juni	VKM og søker har møte for å avklare vesentlige mangler og aspekter knyttet til innholdet i versjon 2 av søknaden
3. juli	VKM gir tilbakemelding til Miljødirektoratet om at søknaden fortsatt har vesentlige mangler (vedlegg 2). Miljødirektoratet iverksetter ny klokkestopp
13. juli	VKM mottar oppdatert søknad (versjon 3 – ny informasjon i blå tekst) via Miljødirektoratet. Klokkestoppen oppheves.
18. juli	Ny versjon (nr. 3) gir fremdeles ikke svar på det som er etterspurt av vesentlige mangler, men VKM gir beskjed til Miljødirektoratet at VKM mener de kan utarbeide en risikovurdering basert på denne versjonen.
1. september	I det videre arbeidet avdekkes ytterligere behov for data og avklaringer. VKM informerer Miljødirektoratet om at det fortsatt foreligger mangler ved søknaden og at usikkerheten i risikovurderingen ansees som veldig høy. For å redusere usikkerheten etterspurte VKM mer informasjon (vedlegg 3). Miljødirektoratet videreformidler VKMs innspill til søker.
7.sept	VKM mottar oppdatert søknad (versjon 4 – ny informasjon i oransje tekst) via Miljødirektoratet (vedlegg 4).