AQUAEXCEL

Aquaculture Infrastructures for Excellence in European Fish Research

Project number: 262336

Combination of CP & CSA
Seventh Framework Programme
Capacities

Deliverable D9.1

Optimization of gynogenesis in salmon

Due date of deliverable: M12 (postponed to M18)

Actual submission date: M20

Start date of the project: March 1st, 2011 **Duration**: 48 months

Organisation name of lead contractor: IMR

Revision: V.1

Project co-funded by the European Commission within the Seventh Framework Programme (2007-2013)	
Dissemination Level	
PU Public	**X**
PP Restricted to other programme participants (including the Commission Services)	
RE Restricted to a group specified by the consortium (including the Commission Services)	
CO Confidential, only for members of the consortium (including the Commission Services)	

Table of contents

Glossary

AQUAEXCEL: Aquaculture Infrastructures for Excellence in European Fish Research

Definitions

Gynogenesis: a form of induced parthenogenesis in which the nuclear DNA of the organism is of maternal origin only. The sperm is irradiated before fertilising the egg, to inactivate the paternal nuclear genome. Diploidy may be restored by suppression of the second meiotic division (leading to “meiotic” gynogenetic, partially heterozygous due to recombination at meiosis) or of mitosis in the haploid zygote (leading to “mitotic” gynogenetic, dihaploid, completely homozygous).

Homozygous clone founder: a completely homozygous individual produced by androgenesis or mitotic gynogenesis from an outbred clone founder. Genetically identical progeny produced from such an individual (by androgenesis or gynogenesis; in subsequent generations, hormonal sex reversal may be used to produce individuals of both sexes to allow propagation by normal crosses) will constitute an isogenic line.

Isogenic line: a group of genetically identical, completely homozygous individuals. Also called **fully inbred clonal line**. In this context, **homozygous clone founders** are produced by induced androgenesis or mitotic gynogenesis from **outbred clone founders**, then these founders are propagated by androgenesis or gynogenesis. In subsequent generations, hormonal sex reversal may be used to produce individuals of both sexes to allow propagation by normal crosses.

Outbred clone founder: an individual used as a source of eggs or sperm to produce a homozygous clone founder by androgenesis or mitotic gynogenesis. Such an individual may come from an outbred population, a defined strain or a line selected for particular traits. Such individuals would be expected to be heterozygous at a proportion of loci.

Appendix 1 illustrates the production of an isogenic line using androgenesis.

Summary

Objectives
The objective of this deliverable was to optimize mitotic gynogenesis as a technique for developing isogenic lines in the Atlantic salmon.

The term “optimize” should be qualified here. Yields of mitotic gynogenetic individuals (as survivors at first feeding stage) are normally low – in general a few percent relative to that observed in control groups (untreated sperm and eggs, normal crosses) – and variable. Some of this variation is due to egg quality (poor egg quality will give disproportionately low yields of mitotic gynogenetics, often zero) and the genetics of the individuals used as outbred clone founders (e.g. recessive lethal or deleterious alleles). In practice, the objective is to be able to produce enough homozygous clone founders to then be able to go on to produce isogenic lines. Homozygous clone founders are completely inbred, so an (unpredictable) proportion will have reduced reproductive capacity.

Rationale
Mitotic gynogenesis (and androgenesis, producing equivalent dihalpoid animals apart from mitochondrial DNA being of maternal origin in androgenetics) has been induced in a wide variety of fish species (reviewed by Komen and Thorgaard, 2007). This includes some salmonids, but there is very little published research on inducing mitotic gynogenesis in the Atlantic salmon (Johnstone and Stet, 1995; Davidson et al., 2010).

The methodology chosen to induce mitotic gynogenesis in Atlantic salmon here was UV irradiation of sperm to inactivate the paternal genome, followed by pressure shock to suppress mitosis.

Main findings
Optimized UV irradiation treatment of sperm produced hatched embryos with typical haploid syndrome, with a very low percentage of embryos of normal appearance. UV irradiation of sperm plus pressure shocks (9500 psi, 5 min. duration) given at 4400-4800 degree-minutes (min^{o}C) postfertilization resulted in surviving embryos (putative mitotic gynogenetics), while shocks given earlier (3800-4200 min^{o}C postfertilization) did not.

Over 300 fish from these treated groups have been reared, PIT tagged and biopsied. Genetic analysis using 15 markers showed that only two of these fish exhibited biparental inheritance (presumably UV inactivation failure), while all other fish were homozygous at all of the 15 markers for which the mother was heterozygous.

These fish are candidate homozygous clone founders.

Teams involved
IMR, UoS

Geographical areas covered
Norway, UK: it is intended to make isogenic Atlantic salmon more widely available.

1. Optimization of mitotic gynogenesis in Atlantic salmon

On 14 December 2011 at IMR, 4 ml of milt from one male salmon was diluted with 160 ml milt fluid (milt from several males were centrifuged until clear and the clear milt fluid was frozen and stored at -20 C and thawed before the experiment). Twelve 15 ml aliquots of the diluted milt were irradiated with UV light for 6 or 8 mins in 8 cm Petri dishes at 480 mW and transferred to 25 ml polyethylene (PE) containers and stored refrigerated and in darkness until fertilization. One control group was made with the diluted unirradiated milt (Table 1).

Table 1. Experimental design and group names.

Milt UV exposure times (min.)		**Group name (min^{o}C shock time/UV exposure time)**	
6	8	4800/6	4800/8
6	8	4600/6	4600/8
6	8	4400/6	4400/8
6	8	4200/6	4200/8
6	8	4000/6	4000/8
6	8	3800/6	3800/8
Control		Control	

The UV irradiation doses used were based on a trial carried out at UoS before the AQUAEXCEL project started (Figs. 1 and 2).

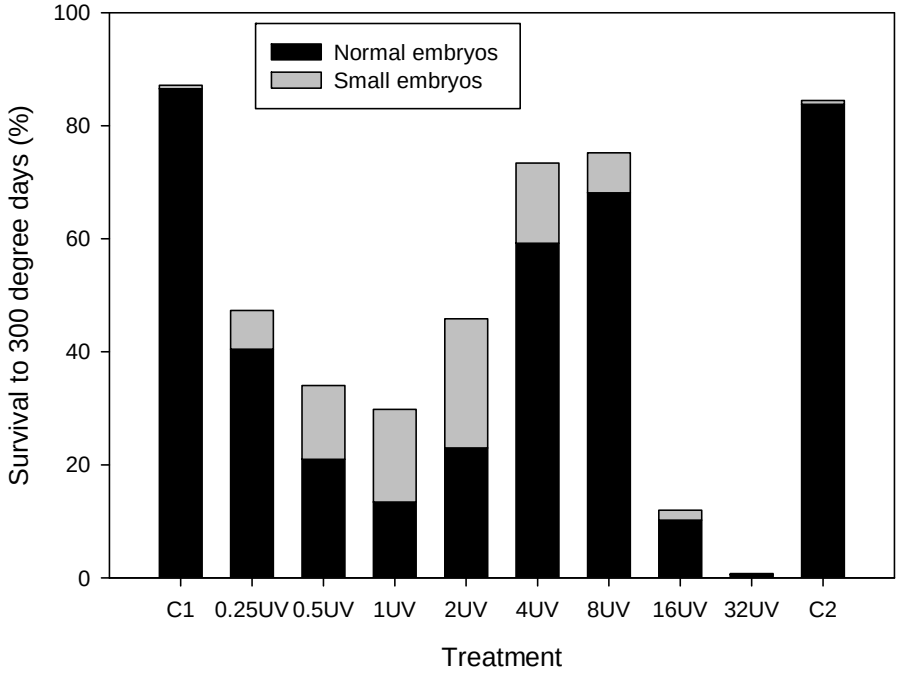


Figure 1. The effect of UV irradiation of sperm on survival of embryos at 300 degree days. C1, C2 = controls (no UV irradiation). UVx = duration of UV irradiation (min). Sperm was diluted to 5 x 10^8 ml^{-1} and irradiated at 170 µW.cm^{-2} with 254nm UV light.

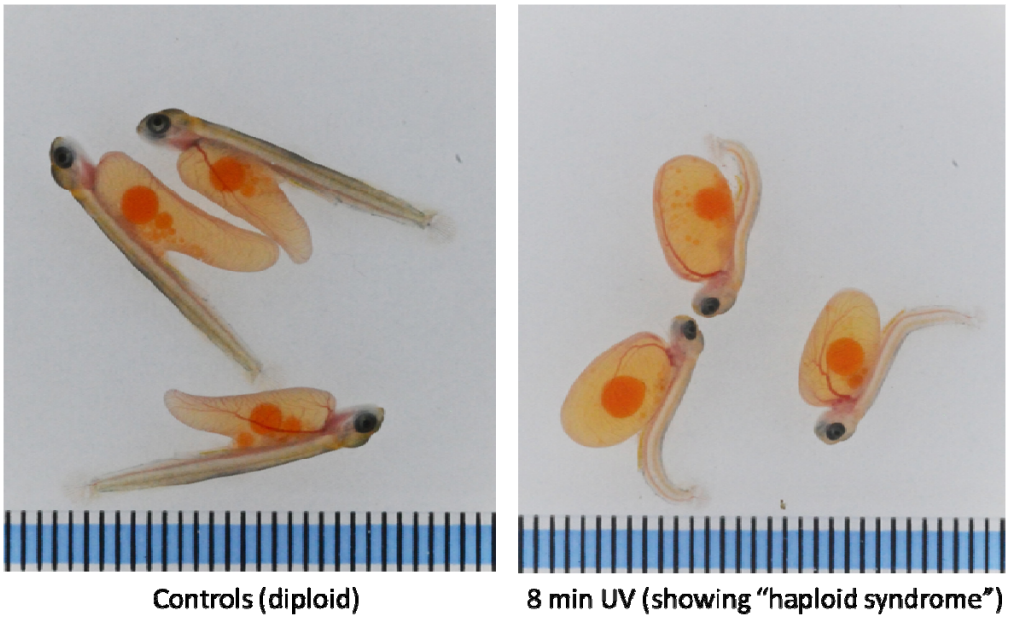


Figure 2. Hatched embryos from control and 8 min UV groups (see Fig 1). Scale bar = 1 mm.

1000 salmon eggs from one female were fertilized with each of the sperm aliquots and left to hydrate in 0.5 L PE bottles at 8 C until pressure treatment. At 3798 (3800), 4000 (4000), 4193 (4200), 4403 (4400), 4605 (4600) and 4816 (4800) min^{o}C the PE bottles were transferred to the pressure chamber and the eggs were pressurized for 5 mins at 9500 psi (The simplified group name is shown in brackets).
At the eyed stage and at hatching the groups were checked for survival (Table 2). No surviving eggs were found in the 3800, 4000 or 4200 group, but surviving eggs were found in all other groups.

Table 2. Survival (percent) in salmon eggs fertilised with UV light irradiated sperm. The survivals on 31 January and 26 March are measured in a sample of c. 20 eggs. The survival to 7 August is based on number of surviving individuals.

Sample date	**UV (mins)**	**Survival (%) at different times for pressure induction (min^{o}C postfertilisation)**					
		3800	4000	4200	4400	4600	4800
31 Jan	6	0	0	0	0	6.25	63.20
	8	0	0	0	4.35	8.70	50.00
26 Mar	6	0	0	0	3.7	4.0	47.6
	8	0	0	0	0	13.4	52.4
7 Aug	6				0	1.2	13.5
	8				0.2	1.7	15.1

2. Genetic analysis of putative mitotic gynogenetics

On 7th August 2012 the surviving fish were PIT tagged and a small fin-clip was taken for genotyping.
DNA was extracted from fin-clips. This was performed in 96-well format using a commercially available extraction kit (Qiagen DNeasy®96 Blood & Tissue Kit). Each 96-well plate included two blank wells as negative controls. All samples were subject to genotyping with a set of 18 microsatellites that are used in the molecular genetics laboratory at the Institute of Marine Research for Atlantic salmon genetics projects (e.g., Glover *et al.* 2010, 2011, 2012). These loci were amplified in three multiplexes, using standard protocols for fresh tissues (full genotyping conditions available from authors upon request); *SSsp3016* (Genbank no. AY372820), *SSsp2210*, *SSspG7*, *SSsp2201*, *SSsp1605*, *SSsp2216* (Paterson *et al.* 2004), *Ssa197*, *Ssa171*, *Ssa202* (O´Reilly *et al.* 1996), *SsaD157*, *SsaD486*, *SsaD144* (King *et al.* 2005), *Ssa289*, *Ssa14* (McConnell *et al.* 1995), *SsaF43* (Sanchez *et al.* 1996), *SsaOsl85* (Slettan *et al.* 1995), *MHC I* (Grimholt *et al.* 2002) and *MHC II* (Stet *et al.* 2002). PCR products were analyzed on an ABI 3730 Genetic Analyzer and sized by a 500LIZ™ size-standard. The raw data was controlled manually twice before export for statistical analysis. The chromosomal position of these markers and thus their power to discriminate mitotic and meiotic gynogenetics is being investigated.

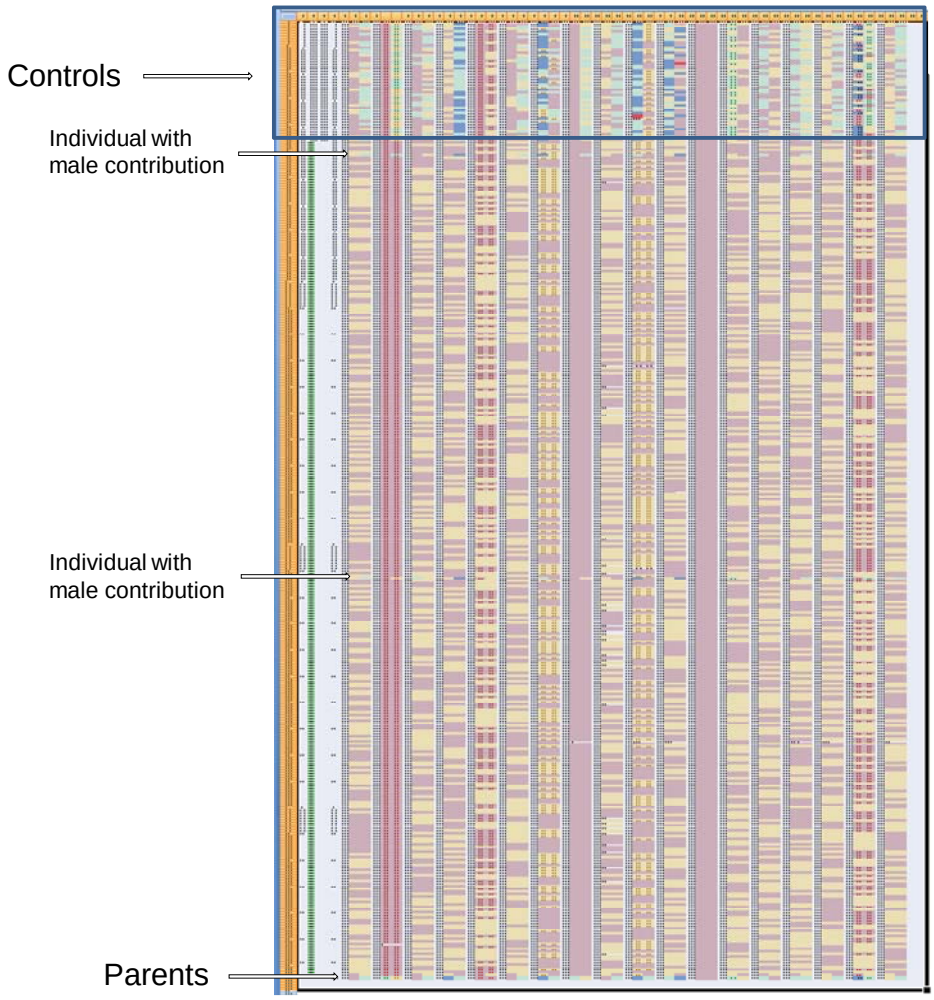


Figure 3. Microsatellite analysis of 2 parents and 362 offspring. The first 45 individuals are fertilized with diluted, but unirradiated milt. The others were fertilized with UV-irradiated milt and pressure shocked. Sire (sperm) alleles are presented in blue and green. Only two individuals from the putative mitotic gynogenetic groups showed any paternal contribution.

After tagging and sampling the experimental fish (including 45 control fish) were transferred to three tanks for further ongrowing. The different experimental groups were distributed among these three tanks for safety reasons.

Table 3. Numbers of surviving individuals in the different groups.

Pressure (minC)	UV irradiation (min.)	
	6	**8**
4400	0	2
4600	12[1]	17
4800	135	151[1]

[1] One fish with male contribution was found in each of these groups.

All the remaining fish will be reared on for further analysis and for phenotypic description. All the individuals without male contribution were homozygous for all the markers where the mother was heterozygous (15 of the markers). These fish will all be candidates for the production of clonal lines.

In October we will start the work to further optimize the protocol and to produce the candidates for the second generation of clonal lines.

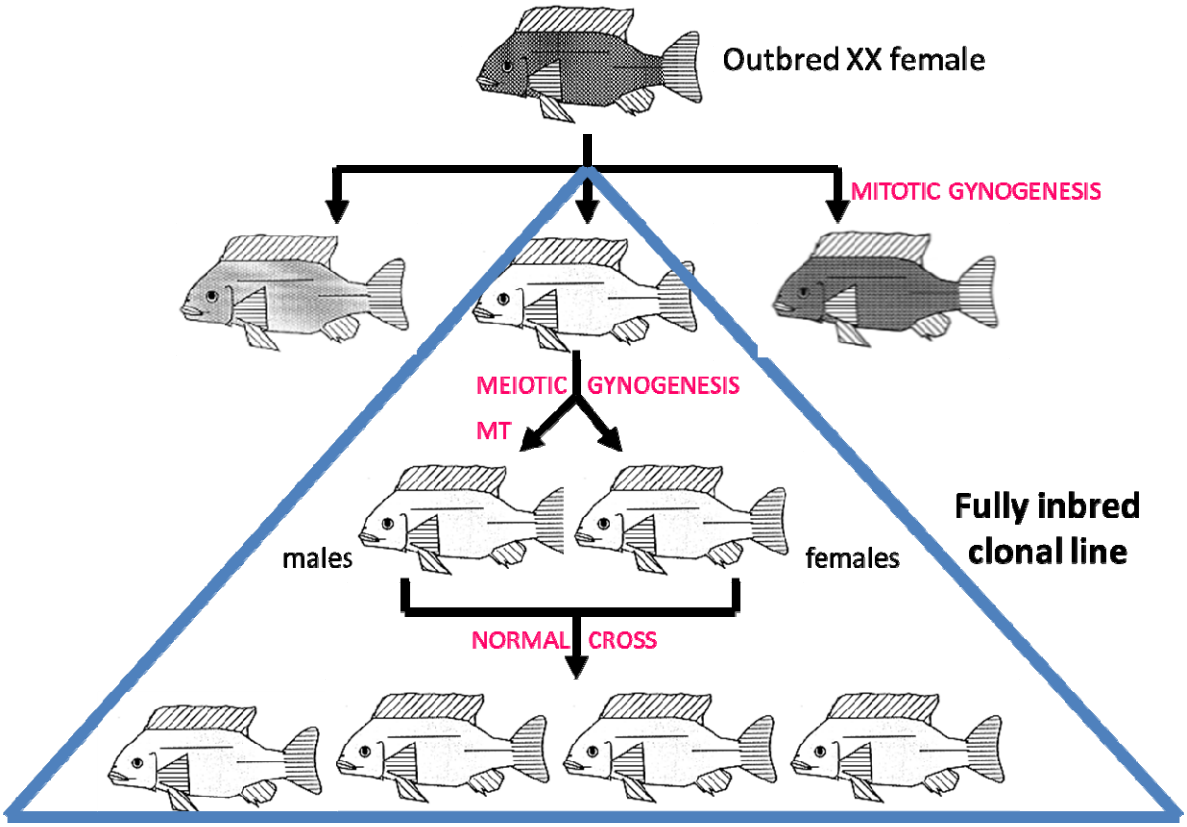
Conclusions

- A series of pressure shocks were given to Atlantic salmon eggs fertilized using UV-irradiated sperm.
- >300 putative mitotic gynogenetic Atlantic salmon were produced from pressure shocks given at 4400-4800 min^{o}C post-fertilization.
- These fish are being grown on as potential isogenic line clone founders.

Appendix 1:

Example of development of an isogenic line, in an XX/XY species via gynogenesis

The diagram below illustrated the development of an XX isogenic line in a species with XX/XY sex determination, using gynogenesis (and hormonal masculinization in later generations). A single outbred clone founder will generate inbred clone founders that differ from each other, reflecting heterozygosity in the outbred clone founder and recombination leading to gamete (egg) production. Thus isogenic lines generated from sibling inbred clonal founders will vary from each other.



MT = masculinization by treatment with 17α-methyltestosterone

References

Davidson WS, Koop BF, Jones SJM, Iturra P, Vidal R, Maass A, Jonassen I, Lien S, Omholt SW. (2010) Sequencing the genome of the Atlantic salmon. Genome Biology **11**:403.

Glover KA, Hansen MM, Lien S, Als TD, Hoyheim B, Skaala O (2010) A comparison of SNP and STR loci for delineating population structure and performing individual genetic assignment. *Bmc Genetics* **11**.

Glover KA, Quintela M, Wennevik V, Besnier F, Sørvik AGE, Skaala O (2012) Three decades of farmed escapees in the wild: A spatio-temporal analysis of population genetic structure throughout Norway. *Plos One* **7**.

Glover KA, Skaala O, Sovik AGE, Helle TA (2011) Genetic differentiation among Atlantic salmon reared in sea-cages reveals a non-random distribution of genetic material from a breeding programme to commercial production. *Aquaculture Research* **42**:1323-31.

Grimholt U, Drablos F, Jorgensen SM, Hoyheim B, Stet RJM (2002) The major histocompatibility class I locus in Atlantic salmon (*Salmo salar* L.): polymorphism, linkage analysis and protein modelling. *Immunogenetics* **54**: 570-81.

Johnstone R, Stet RJM. (1995) The production of gynogenetic Atlantic salmon, Salmo salar L. Theor. Appl. Genet. **90**:819-826.

King TL, Eackles MS, Letcher BH (2005) Microsatellite DNA markers for the study of Atlantic salmon (*Salmo salar*) kinship, population structure, and mixed-fishery analyses. *Molecular Ecology Notes* **5**:130-2.

Komen H, Thorgaard GH. (2007). Androgenesis, gynogenesis and the production of clones in fishes: a review. Aquaculture **269**:150-173.

McConnell SK, Oreilly P, Hamilton L, Wright JN, Bentzen P. (1995) Polymorphic microsatellite loci from Atlantic salmon (*Salmo salar*) - genetic differentiation of North-American and European populations. *Canadian Journal of Fisheries and Aquatic Sciences* **52**:1863-72.

O´Reilly PT, Hamilton LC, McConnell SK, Wright JM (1996) Rapid analysis of genetic variation in Atlantic salmon (*Salmo salar*) by PCR multiplexing of dinucleotide and tetranucleotide microsatellites. *Canadian Journal of Fisheries and Aquatic Sciences* **53**:2292-8.

Paterson S, Piertney SB, Knox D, Gilbey J, Verspoor E (2004) Characterization and PCR multiplexing of novel highly variable tetranucleotide Atlantic salmon (*Salmo salar* L.) microsatellites. *Molecular Ecology Notes* **4**:160-2.

Sanchez JA, Clabby C, Ramos D, Blanco G, Flavin F, Vazquez E, Powell R (1996) Protein and microsatellite single locus variability in *Salmo salar* L (Atlantic salmon). *Heredity* **77**:423-32.

Slettan A, Olsaker I, Lie O (1995) Atlantic salmon, *Salmo salar*, microsatellites at the *SsOSL25, SsOSL85, SsOSL311, SsOSL417* loci. *Animal Genetics* **26**:281-2.

Stet RJM, de Vries B, Mudde K, Hermsen T, van Heerwaarden J, Shum BP, Grimholt U (2002) Unique haplotypes of co-segregating major histocompatibility class II A and class II B alleles in Atlantic salmon (*Salmo salar*) give rise to diverse class II genotypes. *Immunogenetics* **54**:320-31.

Annex 1

Deliverable Check list (to be completed by Deliverable leader)

	Check list		Comments
BEFORE	I have checked the due date and have planned completion in due time		*Please inform Management Team of any foreseen delays (X)*
	The title corresponds to the title in the DOW	X	*If not please inform the Management Team with justification*
	The dissemination level corresponds to that indicated in the DOW	X	
	The contributors (authors) correspond to those indicated in the DOW	X	
	The Table of Contents has been validated with the Activity Leader	X	*Please validate the Table of Content with your Activity Leader before drafting the deliverable*
	I am using the AQUAEXCEL deliverable template (title page, styles etc)	X	*Available in "Useful Documents" on the collaborative workspace*
The draft is ready			
AFTER	I have written a good summary at the beginning of the Deliverable	X	*A 1-2 pages maximum summary is mandatory (not formal but really informative on the content of the Deliverable)*
	The deliverable has been reviewed by all contributors (authors)	X	*Make sure all contributors have reviewed and approved the final version of the deliverable. You should leave sufficient time for this validation.*
	I have done a spell check and had the English verified	X	*Ask a colleague with a good level of English to review the language of the text and do a spell-check too.*
	I have sent the final version to the Activity Leader and to the 2nd Reviewer for approval	X	*Send the final draft to your Activity Leader and the 2nd Reviewer and leave 2 weeks for feedback and final changes before the due date. Once validated by the 2 reviewers, the draft is ready to be sent to the Management Team that will ask for the Coordinator validation and then transfer it to the EC.*