Doc III A section 7.4.3.1.1 (01) Annex Point IIIA XIII 2.1 — Prolonged toxicity to appropriate fish species – effects on biomarkers

5.3	**Conclusion**	The methods used for assessing effects of the test substance on biomarkers in rainbow trout, Atlantic cod and turbot are appropriate and have been described in scientific peer-reviewed literature. Effects on EROD activity were determined after 2, 5, 14 and 17 days of exposure. Inhibition of induced EROD activity was determined after 3 days exposure to β-naphthoflavone. All controls displayed normal EROD activity. The does-response relationships indicate that the test substance have effects on EROD activity starting at 0.001 mg/L or 0.1 mg/kg fish. The stimulation of EROD activity is regarded weak.	X
5.3.1	Other Conclusions	No other conclusions made.	
5.3.2	Reliability	Based on the methods which are in accordance with acceptable scientific principles, the reliability indicator is 2.	X
5.3.3	Deficiencies	Yes The analysed concentrations of test substance were not used for dose-response determination.	

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Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	17/1/13
Materials and Methods	Applicant's version considered acceptable, noting the following: 1.1 It is noted that EROD is the abbreviation used for ethoxyresorufin-O-deethylase activity. 1.2 There is no cross reference to the Handa paper in this report. 3.1.1 The batch number is not given in the published report. 3.1.2 It is not possible to verify that synthesis of medetomidine in the 'Marine Paint Formulation' project is via the methodology given in the Handa paper cited. 3.1.3 The purity of the active substance is not stated in the published report. 3.1.5 Details of solubility with temperature and pH is given in Doc IIIA Section 3. 3.4.2 The report clearly states that the species Psetta maxima is a turbot and not a trout. 3.4.5 The report just states the pH was about 7.5 for the rainbow trout and 8.0 for the Atlantic salmon. **To aid interpretation the report has been examined to clarify exactly when conversion to mg values has been provided in the report this is as follows:** **Injection experiments (report section 2.3)** **Rainbow trout injection: 5.0 µmol (1 mg/kg)** **Atlantic cod injection: 0.5 and 5.0 µmol (0.1 and 1 mg/kg)** For the flow through test system the concentrations are not converted in to mg/L in the report but are instead given as nominal concentrations of 0.05, 0.5, and 5.0 nM respectively for the rainbow trout and 0.5, 5.0 and 50 nM for the Atlantic cod and 0.05, 0.5 and 5.0 nM for the turbot(Section 2.4 of the report and Table A7_4_3_1_1(01)-5). 3.4.5 For the in-vitro experiment the report states that the Atlantic cod and rainbow trout were injected with 50 mg/kg (n=8-10) and turbot with 20 mg/kg (n=6) fish. No further details regarding the test solutions are given. For the sampling of the microsomal fraction it is stated that medetomidine was added to give 10 final concentrations between 0 and 25 µM. The number of fish per experiment is stated to be as described above but it is not totally clear whether this refers to the fish in vivo or injection experiments.

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Prolonged toxicity to appropriate fish species – effects on biomarkers

Results and discussion

Applicant's version considered acceptable ,noting the following:

4.2.1 For the flow through test system the concentrations are not converted in to mg/L in the report but are instead given as nominal concentrations of 0.05 , 0.5 , and 5.0 nM respectively for the rainbow trout and 0.5, 5.0 and 50 nM for the Atlantic cod and 0.05, 0.5 and 5.0 nM for the turbot(Table A7_4_3_1_1(01)-5).

For the injection experiment the report states that the Atlantic cod and turbot were injected with 0.5 and 5.0 µmol (0.1 and 1.0 mg) medetomidine/kg fish and 5.0 µmol (1.0 mg) medetomidine/kg fish for the rainbow trout. No further details regarding the test solutions are given.

For the in vitro experiments for the sampling of the microsomal fraction it is stated that medetomidine was added to give 10 final concentrations between 0 and 25 µM. The number of fish per experiment is stated to be as described above but it is not totally clear whether this refers to the fish in vivo or injection experiments. β-napthoflavone dissolved in peanut oil was injected into the fish (50 mg/kg fish for the Atlantic cod and rainbow trout and 20 mg/kg fish for the turbot).

4.2.3 For rainbow trout there was a significant increase in EROD activity 2 days after injection with 5.0µmol/kg fish. For the flow through study the report results refer to the concentration as 5.0 nM. For the turbot the injection concentrations are referred to as 0.5 and 5.0 nM respectively.

4.2.3 The results presented in the report for the in –vitro inhibition study of EROD activity in mircosomes are as follows:

Table 1
In vitro inhibition study of CYP1A (EROD) activity in fish liver microsomes

Species	No. of assays	Mean IC_{50} (nM)	Standard error
Rainbow trout	5	35	10
Turbot	3	47	17
Atlantic cod	4	111	70

Converting these values to mg/L (assuming 1 nM is 0.0002 mg/L) then the IC50 values are as follows:-

Fish	*IC50 nM*	*IC50 mg/L*
Rainbow trout	*35*	*0.007*
Turbot	*47*	*0.0094*
Atlantic cod	*111*	*0.0222*

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Conclusion

Applicant's version considered acceptable, noting the following:

Please note in the following section that the same units are used as in the report to avoid confusion; so this means that both µmol/kg and nM are used. It should be noted that the UKCA considered that it was important to reflect any trends observed in the data even when these were below statistically derived effect concentrations. This is detailed below.

5.2 As indicated above the report actually refers to some concentrations in terms of nM (see 3.4.5). The 7 fold effect referred to is the maximum observed. In the water exposure there are significant effects on rainbow trout at 5.0 nM ($p<0.05$). The in-vitro inhibition of microsomal EROD activity results are as presented in Table 1 above (results were not converted to mg/L in the report but this has been done above).

5.2.1 NOEC: water exposure; a NOEC for rainbow trout is not presented in the report, a statistically derived significant effect was reported at 5.0 nM and so this constitutes the statistically derived LOEC. When figure 2 of the report is examined it appears that there is a possible effect (non-significant) at 0.05nM and there is a possible dose response to 5.0 nM f1 mg/kg at 2 days from exposure via water. There is a statistically significant effect at 5.0nM after 17 days exposure. The UKCA considers that this means that the statistically derived LOEC is questionable. For injected rainbow trout (5.0 µmol/kg (1 mg/kg)) there was a statistically significant increase in EROD activity after 2 days but effects were comparable with the control after 5 days. The UKCA considers that due to this effect the derivation of a robust NOEC is not possible.

For the Atlantic cod there was no statistically significant effect up to the highest concentration tested of 50 nM (0.01 mg/L) via water exposure, The report indicates that there were no statistically significant effects from injection at either 0.5 or 5.0 µM. However when figure 5 of the report is examined at 2 days there is an increase in EROD activity post-injection at 5.0 µM., the level being approximately 0.36 nmol/mg protein/min compared with 0.2 nmol/mg protein/m in the control (Figure 5). After 5 days the levels in the control and treatments are similar. Levels were fairly similar to the control at 0.5 µmol/kg at both day 2 and 5. On balance the UKCA considers that if the effect at 5.0 µM /kg is taken into account the NOEC is likely to be 0.5 µM/kg (0.1 mg/kg) even though this is not statistically significant.

For turbot via water exposure the EROD activity was lower at all concentrations and this includes the lowest concentration of 0.05 nM than in the control (figure 4). This inhibition was not significant and so the statistically derived LOEC would be 5.0 nM. However, the data were variable and the UKCA questions is it is possible to derive a robust LOEC (a LOEC is not reported). For injected fish there was a statistically different EROD activity at both 0.5 and 5.0 µmol/kg fish (figure 3) at day 2, differences were not significant at day 5.

5.2.2 LOEC: For injected rainbow trout the results are referred to as 5.0µmol/kg fish rather than 1 mg/kg. In the water exposure a possible trend in effect was seen at 0.05 nM (figure 2 as detailed above) although this effect was not statistically significant. For injected turbot effects were seen at 0.5 µmol/kg the lowest concentration tested. For turbot via water exposure the effects were not statistically significant but it is noted that the data were variable and therefore the reliability of the NOEC is questionable. For Atlantic cod for water exposure the LOEC was above 50 nM (0.01 mg/L; the highest concentration tested). For injection the reliability of the statistically derived LOEC of 5.0 µM/kg is questioned.

5.2.3 The IC50 values given in the report are presented in Table 1 above.

5.3 See comments relating to all sections 5.2 above.

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Prolonged toxicity to appropriate fish species – effects on biomarkers

Reliability	4
Acceptability	Not acceptable
Remarks	There is no agreed protocol for this testing procedure. The study was not undertaken to GLP. It could not be verified that the medetomidine was produced as per the Handa paper as no cross reference was given therefore full details of the purity of the medetomidine etc could not be verified. Additionally there were issues with regard to the conversion of the end points from nM to mg as only for some of the values were both forms of units presented in the report. It was not possible to verify the concentrations of medetomidine used in the water exposure studies as the results were unreliable and results were simply based on nominal concentrations. Nor was analytical verification of the nominal concentrations provided. Also as this is a published report the raw data could not be accessed to tease out and confirm the information in the report or to clarify some points which were not covered or were unclear. It is considered that there is considerable uncertainty attached to this study and it is considered unreliable.
	COMMENTS FROM ... *(specify)*
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Doc III A section 7.4.3.1.1 (01) Annex Point IIIA XIII 2.2 — Prolonged toxicity to appropriate fish species – effects on biomarkers

Table A7_4_3_1_1(01)-1:Preparation of TS solution for poorly soluble or volatile test substances

Criteria	Details
Dispersion	No
Vehicle	KCl (injection), Methanol p.a (flow-through)
Concentration of vehicle	KCl 10 ml/kg fish, Methanol 0.00017%
Vehicle control performed	Yes – all treatments exposed to the same concentrations of vehicle.
Other procedures	No

Table A7_4_3_1_1(01)-2:Dilution water

Criteria	Details
Source	Rainbow trout: Dechlorinated, aerated tap water. Atlantic cod: Natural sea water from a 50 m deep drill hole in a coastal lava field, Sandgerði, Iceland for injection and flow-through test systems. Artificial seawater for in vitro test system. Turbot: Natural sea water from a 50 m deep drill hole in a coastal lava field, Sandgerði, Iceland.
Salinity	Rainbow trout: No salinity Atlantic: 32-33 ppt (injection and flow-through), 20 ppt (in vitro) Turbot: 32-33 ppt
Hardness	Not measured
pH	Rainbow trout: 7.5 Atlantic cod and Turbot: 8.0
Oxygen content	Not measured
Conductance	Not measured
Holding water different from dilution water	No

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Table A7_4_3_1_1(01)-3:Test organisms

Criteria	Details
Species/strain	Rainbow trout (*Oncorhynchus mykiss*), Atlantic cod (*Gadus morhua*) and Trout (*Psetta maxima*)
Source	Rainbow trout: Antens laxodling AB (Alingsås, Sweden) Atlantic cod: Hafrannsóknastofnunin (Stadur, Iceland) for injection and flow-through test systems, Swedish west coast for in vitro experiment Turbot: Sæbýli HF fiskeldi (Vógavík, Vogar, Iceland)
Wild caught	Atlantic cod used for in vitro experiment
Age/size	Juveniles were used Rainbow trout: 20-50 g Atlantic cod: 10 g (injection), 70 g (flow-through) Turbot: 100 g
Kind of food	Rainbow trout: Commercial pellets Atlantic cod and turbot: No feeding
Amount of food	No information
Feeding frequency	Twice a week during acclimatisation
Post-hatch transfer time	Fish bought as juveniles
Time to first feeding	Fish bought as juveniles
Feeding of animals during test	No
Treatment for disease within 2 weeks preceding test	No information available

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Table A7_4_3_1_1(01)-4: Injection Test system

Criteria	Details
Test type	Injection test system
Test solution	Rainbow trout: 0 (control) and1.0 mg/kg fish test substance in 0.15 M KCl Atlantic cod: 0 (control), 0.1 and 1.0 mg/kg fish test substance in 0.15 M KCl Turbot: 0 (control), 0.1 and 1.0 mg/kg fish test substance in 0.15 M KCl
Renewal of test solution	No
Volume of test vessels	Rainbow trout: 50 L Atlantic cod: 30 L Turbot: 200 L
Volume/animal	Rainbow trout: 7.1 L Atlantic cod: 3 L Turbot: 9 L
Number of animals/vessel	Rainbow trout: 7 Atlantic cod: 10 Turbot: 22
Number of vessels/ concentration	No vessel / concentration, fish exposed with injections.
Test performed in closed vessels due to significant volatility of TS	No

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Table A7_4_3_1_1(01)-5: Flow-through Test system

Criteria	Details
Test type	Flow-through test system
Test solution	Rainbow trout and Turbot: 0 (control), 0.00001 mg/L, 0.0001 mg/L and 0.001 mg/L Atlantic cod: 0 (control), 0.0001 mg/L, 0.001 mg/L and 0.01 mg/L
Renewal of test solution	Rainbow trout: water flow 500 ml/min, test substance 50 µl/min Atlantic cod and turbot: water flow 300 ml/min, test substance 50 µl/min.
Volume of test vessels	Rainbow trout: 50 L Atlantic cod: 60 L Turbot: 60 L
Volume/animal	Rainbow trout: 4.5 L Atlantic cod: 5 L Turbot: 5 l
Number of animals/vessel	Rainbow trout: 11 Atlantic cod: 12 Turbot: 12
Number of vessels/ concentration	1
Test performed in closed vessels due to significant volatility of TS	No

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Table A7_4_3_1_1(01)-6: In vitro Test system

Criteria	Details
Test type	In vitro experiment using liver microsomes from β-naphthoflavone exposed fish
Test solution	Injection of β-naphthoflavone; Rainbow trout and Atlantic cod 50 mg/kg fish, Turbot 20 mg/kg fish Test substance 0 (control), 0.0008 mg/L, 0.002 mg/L, 0.007 mg/L, 0.02 mg/L, 0.06 mg/L, 0.19 mg/L and 0.56 mg/L.
Renewal of test solution	No
Volume of test vessels (exposed fish)	Rainbow trout: 50 L Atlantic cod: 50 L Turbot: 200 L
Volume/animal	Rainbow trout: 7.1 L Atlantic cod: 7 L Turbot: 9 L
Number of animals/vessel	Rainbow trout: 7 Atlantic cod: 7 Turbot: 22
Number of vessels/ concentration	Not applicable. End points after in vitro measurements.
Test performed in closed vessels due to significant volatility of TS	No

Table A7_4_3_1_1(01)-7: Test conditions

Criteria	Details
Test temperature	10 - 12 °C
Dissolved oxygen	Not measured
pH	7.5-8.0
Adjustment of pH	No
Aeration of dilution water	Yes
Intensity of irradiation	Not measured
Photoperiod	12 h daily

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Prolonged toxicity to rainbow trout (*Oncorhynchus mykiss*) – effects on physiology

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted] The effects of Medetomidine, a new antifouling agent, on rainbow trout physiology. (Unpublished)	X
1.2	**Reference**	[redacted] Medetomidine – effects on pigmentation and melanophore function in rainbow trout after long-term exposure. [redacted] (Unpublished)	
1.3	**Reference**	Handa P, Fant C and Nyden M. (2006) Antifouling agent release from marine coatings – ion pair formation/dissolution for controlled release. Prog. Org. Coat. **57** (4):376-382. (Published) **NEW PUBLISHED PAPER (AUGUST 2013)** Lennquist A, Martensson Lindblad L, Hedberg D, Kristiansson E, Forlin L Colour and melanophore function in rainbow trout after long term exposure to the new antifoulant medetomidine. Chemosphere 80 (2010) 1050-1055 (published).	
1.4	**Data protection**	Yes (unpublished) No (published)	
1.4.1	Data owner	I-Tech AB (unpublished) Public domain (published)	
1.4.2	Criteria for data protection	Data on new a.s. for first approval / authorisation	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	No	
2.2	**GLP**	No Study performed according to scientific sound methods.	
2.3	**Deviations**	Yes Please refer to section 3.4	
		3 METHOD	
3.1	**Test material**	As given in references 1.3	X
3.1.1	Lot/Batch number	No information of batch number available.	
3.1.2	Specification	Medetomidine and medetomidine hydrochloride were used. Please refer to reference 1.3 (Handa et al 2006) and document IIIA, section 2 for further information.	X
3.1.3	Purity	[redacted] %	X
3.1.4	Composition of Product	Crystalline powder	
3.1.5	Further relevant	Properties for medetomidine hydrochloride	

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Prolonged toxicity to rainbow trout (*Oncorhynchus mykiss*) – effects on physiology

	properties	Molecular weight 236.7 g/mol Solubility 24 g/l pKa: 7.1	
3.1.6	Method of analysis	Medetomidine was analysed by NMR, please refer to reference 1.2 for details.	
3.2	**Preparation of TS solution for poorly soluble or volatile test substances**	Methanol was used for preparation of stock solutions. For details see table A7_4_3_1_2(01)-1.	
3.3	**Reference substance**	No reference substance used.	
3.3.1	Method of analysis for reference substance	No reference substance used.	
3.4	**Testing procedure**		
3.4.1	Dilution water	For details on dilution water see table A7_4_3_1_2(01)-2.	X
3.4.2	Test organisms	For details on test organism see table A7_4_3_1_2(01)-3.	X
3.4.3	Handling of test organisms	1 week acclimatisation time prior test	X
3.4.4	Test system	For details on test type, renewal of TS solution etc. see table A7_4_3_1_2(01)-4	
3.4.5	Test conditions	For test conditions see table A7_4_3_1_2(01)-5.	
3.4.6	Duration of the test	17 (25) and 54 days	
3.4.7	Test parameter(s)	Hemoglobin, Lactate, Glucose, Red blood cells, Hematocrit value, Liver size, Growth hormones (GH), Insulin like growth factor I (IGF-I), Pigmentation, Melanophore function and Melanophore density.	
3.4.8	Examination / Sampling	After 17 days sampling of livers and blood were performed (n=10-11). Test organisms were sacrificed with a sharp blow to the head and blood was drawn from the caudal vein. Hemoglobin, glucose content, red blood cells and lactate were analysed immediately. Plasma was separated and stored for GH and IGF- analyses. The liver somatic index was calculated ((liver weight/total body weight x 100). One replicate per treatment was kept until day 25 for measurement of melanophore function. Examination of pigmentation at day 31 and 54, sampling of scales for melanophore analyses at day 54 of the 54 days exposure.	
3.4.9	Monitoring of TS concentration	Yes	
3.4.10	Statistics	Differences between treatments were tested for significance (p=0.05) using Kruskal-Wallis test and Mann-Whitney U-test.	

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Annex Point IIIA XIII 2.1

Prolonged toxicity to rainbow trout (*Oncorhynchus mykiss*) – effects on physiology

4 RESULTS

4.1	**Range finding test**	No range finding test performed	
4.1.1	Concentrations	No range finding test performed	
4.1.2	Number/ percentage of animals showing adverse effects	No range finding test performed	
4.1.3	Nature of adverse effects	No range finding test performed	
4.2	**Results test substance**		
4.2.1	Initial concentrations of test substance	17 (25) days exposure study: 0 (control), 0.00001 mg/L, 0.0001 mg/L and 0.001 mg/L 54 days exposure study : 0 (control), 0.0001 mg/L and 0.001 mg/L	X
4.2.2	Actual concentrations of test substance	Due to analytical difficulties, the data from chemical analyses were not reliable. Therefore, nominal concentrations were used for dose-response determinations and statistics.	
4.2.3	Effect data	17 (25) days exposure study: No significant effects on hemoglobin, lactate, glucose, red blood cells or hematocrit. Fish exposed to 0.0001 mg/L had significantly higher liver somatic index but this was not observed in the 0.001 mg/L treatment. There were no significant changes in blood plasma concentrations of growth hormones or insulin like growth factor I. All individuals exposed to 0.001 mg/L were paler than control fish. There was also a dose-dependent decrease in sensitivity to the test substance with significant effects at 0.001 mg/L when examining melanophore functions. 54 days exposure study: Pigmentation in the test organisms were significantly affected after 31 days of exposure to 0.0001 mg/L. After 54 days of exposure, there was only a significant effect at 0.001 mg/L. The melanophores were significantly more aggregated after 54 days exposure to 0.0001 mg/L but there were no significant effects on melanophore function. There were no differences in number of melanophores between the treatments.	X
4.2.4	Concentration / response curve	Please refer to reference 1.1 and 1.2	
4.2.5	Other effects	No other effects observed.	
4.3	**Results of controls**		
4.3.1	Number/ percentage of animals showing adverse effects	No adverse effects were observed in controls.	

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Annex Point IIIA XIII 2.1

Prolonged toxicity to rainbow trout (*Oncorhynchus mykiss*) – effects on physiology

4.4	**Test with reference substance**	No reference substance used.	
4.4.1	Concentrations	No reference substance used.	
4.4.2	Results	No reference substance used.	

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1	**Materials and methods**	Effects of long-term exposure to the test substance were investigated in rainbow trout. The test organisms were exposed to the test substance in flow-through test systems during 17 (25) and 54 days. Several physiological parameters were measured ; hemoglobin, lactate, glucose, red blood cells, hematocrit value, liver somatic index, growth hormones, insulin like growth factor I, pigmentation, melanophore function and melanophore density.	
5.2	**Results and discussion**	No significant effects of the test substance were observed on hemoglobin, lactate, glucose, red blood cells, hematocrit value, growth hormones or insulin like growth factor I. There was a small increase in liver somatic index at 0.0001 mg/L but not seen at the higher concentration 0.001 mg/L. However, no effects in metabolism or hepatic enzymes were observed. Pigmentation of rainbow trout was affected after 25 and 54 days of exposure to 0.001 mg/L. After 25 days the melanophores aggregated less when exposed to 0.001 mg/L which indicate that rainbow trout were desensitised after long-term exposure. After 54 days there were no indications of desensitisation, the melanophore function in all treatments was normal. No differences in number of melanophores were observed. These results signify that long term exposure to the test substance does not alter melanophore function or induce liver apoptosis.	X
5.2.1	NOEC	0.0001 mg/L (pigmentation 54 days)	X
5.2.2	LOEC	0.001 mg/L (pigmentation 54 days)	X
5.3	**Conclusion**	The methods used for assessing effects of the test substance on physiology in rainbow trout have been described in scientific peer-reviewed literature. Effects on physiological parameters were determined after 17, 25, 31 and 54 days of exposure. All controls displayed normal physiology. The does-response relationships indicate that the test substance have effects on pigmentation starting at 0.001 mg/l after 54 days of exposure. No changes in melanophore functions were observed after 54 days of exposure.	X
5.3.1	Other Conclusions	No other conclusions made.	
5.3.2	Reliability	Based on the methods which in accordance to acceptable scientific principles the reliability indicator is 2.	X

Doc III A section 7.4.3.1.2 (01)
Annex Point IIIA XIII 2.1

Prolonged toxicity to rainbow trout (*Oncorhynchus mykiss*) – effects on physiology

5.3.3	Deficiencies	Yes The analysed concentrations of test substance were not used for dose-response determination.

Doc III A section 7.4.3.1.2 (01) Annex Point IIIA XIII 2.1	**Prolonged toxicity to rainbow trout (*Oncorhynchus mykiss*) – effects on physiology**

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	28/2/13
Materials and Methods	Applicant's version considered acceptable, noting the following: 1.1 The report is undated. 3.1, 3.1.2 Test material: neither report has a cross reference to the Handa report and therefore the method of manufacture of the test material and its specification cannot be verified. The reports instead refer to synthesis via the Marine Paint Formulation project but as indicated it cannot be verified that this is as per the Handa paper. 3.1.2Reference 1.1 refers just to testing with medetomidine whilst in study reference 1.2 medetomidine HCL is referred to. 3.1.3 There is no confirmation of the purity of the active substance used in reference 1.1. 3.4.1. Report reference 1.2 simply states that ultra pure water was used for testing and no other details are provided regarding the dilution water. 3.4.2 Details of the test organism are not provided in report reference 1.2. 3.4.3 An acclimatisation period is not provided in report reference 1.2.

Doc III A section 7.4.3.1.2 (01) Annex Point IIIA XIII 2.1

Prolonged toxicity to rainbow trout (*Oncorhynchus mykiss*) – effects on physiology

Results and discussion

Applicant's version considered acceptable, noting the following:

4.2.1. The 17 (25) day period referred to is for report reference 1.1 and the 54 day period is for report reference 1.2. This report also included a 31 day observation for pigmentation with fish also paler at 31 days after exposure to 0.0001 mg/L. The exposure concentrations given in report reference 1.1 are also given in terms of nM and are 0.05, 0.5 and 5.0 nM respectively. The UKCA confirms these concentrations are 0.00001, 0.0001, 0.001 mg/L.

4.2.3 Reference 1.1 although there was no significant effect on blood plasma or growth hormones there was a trend showing increased growth hormones and decreasing insulin like growth factor with increasing medetomidine concentrations. In terms of pigmentation there were no visual differences in fish colour between the control and the fish exposed to 0.5 nM (0.1 µg/L) and 0.05nM (0.01 µg/L). Report 1.2 also included a 31 day observation for pigmentation with fish also paler at 31 days after exposure to 0.0001 mg/L. At 0.0001 mg/L initially melanophores were significantly more aggregated than melanophores from control fish but the response to msh incubation was dispersion. Thus it was concluded that the melanophores from all treatments were functional despite initial differences in aggregation.

5.2 Reference 1.2: After 54 days exposure the melanophore index was significantly reduced at 0.001 mg/L. There was no significant effect at 54 days at 0.0001 mg/L. Reference 1.1: The report states that there was a dose dependent decrease in sensitivity to medetomidine which was significant at the highest dose (1 µg/L). This meant that melanophores in scales from fish exposed to medetomdine did not aggregate as much (despite the initial aggregation) as the melanophores from control fish in response to further medetomidine administration).

5.2.1 In the [redacted] (reference 1.1) a significant effect on melanophore index was observed after 25 days at 5 nM (0.001 mg/l) and thus the NOEC after 25 days was 0.5 nM (0.0001 mg/l). The NOEC was based on nominal concentrations. It was noted that despite the effect on paleness the function of the melanophores was normal and no apoptosis was observed after 54 days. In report 1.2 by [redacted] it is clearly stated that rainbow trout were significantly paler after both 31 and 54 days at 0.0001 mg/L and that this coincides with previous observations made after 17 days. However, the figures accompanying this statement do not show a significant effect on pigmentation after 54 days at 0.0001 mg/l (these figures are now included below) but only at 0.001 mg/L.

5.2.2 This is also the LOEC after 25 days. The LOEC was based on nominal concentrations.

In conclusion the NOECs and LOECs are considered by the UKCA to be as follows:

Study	NOEC	LOEC
Reference 1.1 Lennquist, martensson et al.	0.0001 mg/ L after 25 days exposure	0.001 mg/L after 25 days exposure
Reference 1.2 Lennquist,Ohlauson	<0.0001 mg/L after 31 days exposure At 54 days the text indicates a significant effect at 0.0001 mg/L but this is NOT shown on the figure presented (see below.	<0.0001 mg/L after 31 days exposure At 54 days the text indicates a significant effect at 0.0001 mg/L but this is NOT shown on the figure presented (see below.

5.3 Effects on pigmentation were also observed stated to be observed in earlier studies after just 17 days.

Doc III A section 7.4.3.1.2 (01) Annex Point IIIA XIII 2.1

Prolonged toxicity to rainbow trout (*Oncorhynchus mykiss*) – effects on physiology

Conclusion	Applicant's version considered acceptable, noting the following: 5.3 Effects on pigmentation were also stated to be observed in earlier studies after just 17 days. It is also noted that the NOEC for pigmentation was dependent on the study and in reference 1.1 was 0.0001 mg/L (25 days exposure) but was <0.0001 mg/L at 31 day in Reference 1.2. At 54 days the text and the figures differ with regards to whether the effect was significant or not at this concentration. There was a significant effect at 0.001 mg/L at 54 days.
Reliability	4
Acceptability	Reference 1.1: Non acceptable Reference 1.2: Non-acceptable
Remarks	Reference 1.1. The report was not undertaken to GLP and the batch number and full details for the medetomidine tested were not available. Additionally raw data were not available to allow scrutiny and verification of the results presented. There was a problem with the analysis of the medetomidine concentrations due to unknown technical reasons and the obtained data were considered unreliable. No statistical analysis of results was presented. Reference 1.2 The report was not undertaken to GLP. Although more details on the active substance were provided than in reference 1.1 a batch number was not provided. Additionally raw data were not available to allow scrutiny and verification of the results presented and issues with the interpretation of the results are highlighted above. Test concentration validation was not provided and it not stated that this was undertaken. No statistical analysis of results was presented.

Doc III A section 7.4.3.1.2 (01) Annex Point IIIA XIII 2.1

Prolonged toxicity to rainbow trout (*Oncorhynchus mykiss*) – effects on physiology

Update August 2013

The company have now provided a published paper (2010, which is stated to summarise the data that were provided in these reports. The title of this paper is' Colour and melanophore function in rainbow trout after long term exposure to the new antifoulant medetomidine. A. Lennquist, L. Martensson Lindblad, D. Hedberg, E. Kristiansson, L. Forlin (Chemosphere 80 (2010) 1050-1055). The full published report is now included in document IV (IV Ecotoxicological profile) report IV-A7-4-3-1-2(04). The key findings are summarised as follows:

Paleness: The report states that fish from the 5nM treatment were significantly paler at both sampling occasions. At the first sampling the fish from the 0.5 nM treatment were also significantly paler than the control fish. Differences in paleness were easily observed in the live fish and both skin and eyes were paler than control fish. Figure 4 shows there was a significant effect at 0.5 nM at 31 days but not at 54 days (this is stated to indicate that desensitisation occurs over time). Significant effects were seen at both 31 days at 5.0nM.

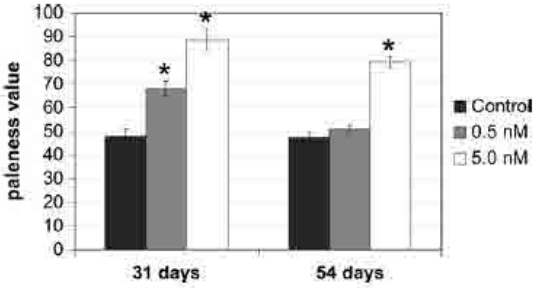


Fig. 4. Paleness evaluated using the ImageJ software. The fish were photographed after 31 and 54 d of exposure. Error bars indicate standard error, * indicate significance when $p \leq 0.05$. $N = 13-28$ fishes.

Melanophore function: Figure 5 shows that melanophores exposed to 5nM medetomidine initially aggregated more than control fish. Addition of melanophore stimulating hormone (MSH) and then medetomidine caused aggregation again. The melanophores were considered functional i.e. pigment dispersal in response to MSH and aggregation in response to medetomidine.

Melanophore density: the number of melanophores did not differ between treatments.

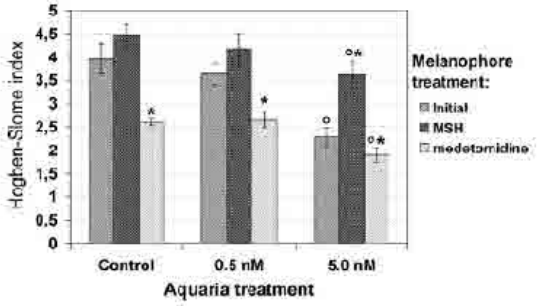


Fig. 5. Melanophore function evaluated using the Hogben–Slome index. Scales from fish exposed to different concentrations of medetomidine before and after administration of melanophore stimulating hormone (MSH) and medetomidine. Error bars indicate standard error. $N = 7-8$ fishes. * indicate significance within each aquaria treatment after *in vitro* melanophore treatment, $p < 0.05$. ° indicate significance among the aquaria treatments when comparing the medetomidine treatments to control, $p < 0.05$.

Doc III A section 7.4.3.1.2 (01) Annex Point IIIA XIII 2.1	Prolonged toxicity to rainbow trout (*Oncorhynchus mykiss*) – effects on physiology
	The UKCA considers that 0.5nM is equivalent to 0.0001 mg/L and 5 nM is equivalent to 0.001 mg/L.
Remarks	This study was not undertaken to GLP and a batch number was not provided for the sample of medetomidine tested. As indicated above the raw data were not available for verification of results and due to unforeseen technical issues with the chemical analyses there were no reliable measures of the concentrations in water from this experiment. The reliability score for this published summary remains a score of 4.
	COMMENTS FROM ... *(specify)*
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub) heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Doc III A section 7.4.3.1.2 (01) Annex Point IIIA XIII 2.2 — Prolonged toxicity to appropriate fish species – effects on biomarkers

Table A7_4_3_1_2(01)-1:Preparation of TS solution for poorly soluble or volatile test substances

Criteria	Details
Dispersion	No
Vehicle	Methanol p.a
Concentration of vehicle	0.01 µl/L
Vehicle control performed	Yes – all treatments were exposed to the same concentrations of vehicle.
Other procedures	No

Table A7_4_3_1_2(01)-2:Dilution water

Criteria	Details
Source	Dechlorinated, aerated tap water.
Salinity	No salinity in tap water
Hardness	Not measured
pH	7.5
Oxygen content	Not measured
Conductance	Not measured
Holding water different from dilution water	No

Table A7_4_3_1_2(01)-3:Test organisms

Criteria	Details
Species/strain	Rainbow trout (*Oncorhynchus mykiss*)
Source	Antens laxodling AB (Alingsås, Sweden)
Wild caught	No
Age/size	Juvenile, 20-50 g
Kind of food	Commercial pellets
Amount of food	No information
Feeding frequency	Twice a week during acclimatisation
Post-hatch transfer time	Fish supplied as juveniles
Time to first feeding	Fish supplied as juveniles
Feeding of animals during test	No
Treatment for disease within 2 weeks preceding test	No information available

Doc III A section 7.4.3.1.1 (01) Annex Point IIIA XIII 2.2 — Prolonged toxicity to appropriate fish species – effects on biomarkers

Table A7_4_3_1_2(01)-4: Flow-through Test system

Criteria	Details
Test type	Flow-through test system
Test solution	17 (25) days exposure: 0 (control), 0.00001 mg/L, 0.0001 mg/L and 0.001 mg/L 54 days exposure: 0 (control), 0.0001 mg/L and 0.001 mg/L
Renewal of test solution	Water flow 500 ml/min, Test substance 50 µl/min
Volume of test vessels	50 L
Volume/animal	4.2 L
Number of animals/vessel	11-12
Number of vessels/ concentration	1
Test performed in closed vessels due to significant volatility of TS	No

Table A7_4_3_1_2(01)-5: Test conditions

Criteria	Details
Test temperature	10 - 12 °C
Dissolved oxygen	Not measured
pH	7.5
Adjustment of pH	No
Aeration of dilution water	Yes
Intensity of irradiation	Not measured
Photoperiod	12 h daily

Figure A7-4-3-1-2(01)-1 Reference 1.2: Results for the 'paleness value' analysed from pictures of rainbow trout exposed to 0.0001 or 0.001 mg/L of test substance. Error bars indicate standard error and *indicate significance (p=0.05)

Figure 1: "Paleness value" analysed from pictures of rainbow trout exposed to 0.0001 or 0.001 mg/l of test substance. Error bars indicate standard error and * indicate significance (p=0.05).

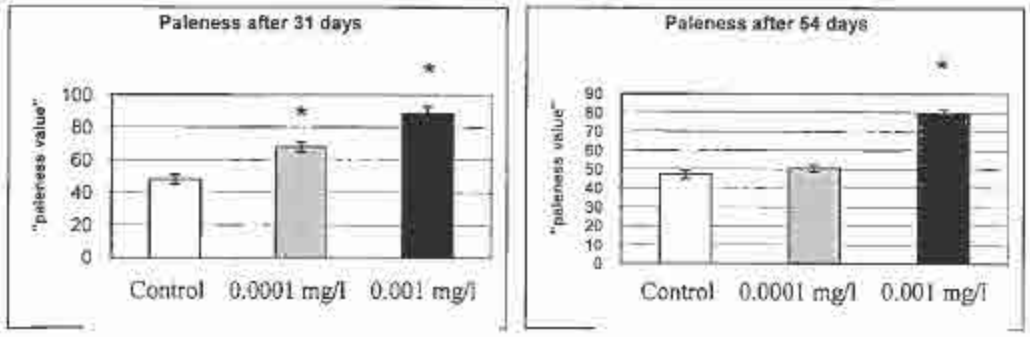
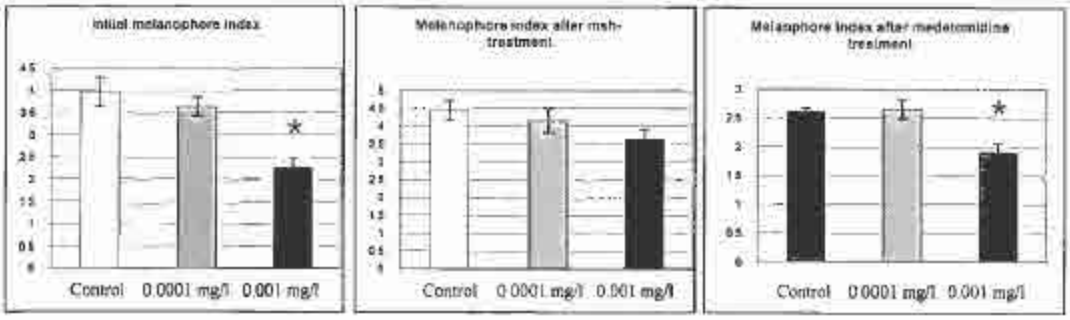


Figure A7-4-3-1-2(01)-2 Reference 1.2: Melanophore index after different treatments. Error bars indicate standard error and *indicate significance (p =0.05)

Figure 2: Melanophore index after different treatments. Error bars indicate standard error and * indicate significance (p=0.05)



Doc III A section 7.4.3.2.1 (01)
Annex Point IIIA XIII 2.2

Effects on reproduction and growth rate in Zebra fish (*Danio rerio*)

1 REFERENCE

1.1	**Reference**	[redacted] Ecotoxicological evaluation of medetomidine hydrochloride – effects on embryo and larvae of Zebra fish. [redacted] (Unpublished)	X
1.2	**Data protection**	Yes	
1.2.1	Data owner	I-Tech AB	
1.2.2	Criteria for data protection	Data on new a.s for first approval /authorisation	

2 GUIDELINES AND QUALITY ASSURANCE

2.1	**Guideline study**	Yes OECD TG 212.	X
2.2	**GLP**	No	
2.3	**Deviations**	No	

3 METHOD

3.1	**Test material**	Medetomidine hydrochloride	
3.1.1	Lot/Batch number	-	X
3.1.2	Specification	Medetomidine hydrochloride, used instead of medetomidine due to higher solubility in water. Final form in water is pH dependent.	
3.1.3	Purity	[redacted] %	X
3.1.4	Composition of Product	Crystalline powder.	
3.1.5	Further relevant properties	Molecular weight: 236.7 g/mol Solubility: 24 g/l pKa: 7.1	X
3.1.6	Method of analysis	Please refer to section IIIA.4.2 for further information.	X
3.2	**Preparation of TS solution for poorly soluble or volatile test substances**	Not performed	
3.3	**Reference substance**	No reference substance used.	
3.3.1	Method of analysis for reference substance	No reference substance used.	

3.4	**Testing procedure**		
3.4.1	Dilution water	Details on dilution water see table A7_4_3_2_1(01)-1.	X
3.4.2	Test organisms	Details on tested organisms see table A7_4_3_2_1(01)-2.	X
3.4.3	Handling of embryos and larvae (OECD 212)	Handling of embryos and larvae were performed according to OECD guideline 212.	
3.4.4	Test system	See table A7_4_3_2_1(01)-3 and A7_4_3_2_1(01)-4 for details.	X
3.4.5	Test conditions	Details on test conditions see table A7_4_3_2_1(01)-5	X
3.4.6	Duration of the test	24 h for egg/embryo development, a minimum of 90% starvation in controls for embryo/fry development.	
3.4.7	Test parameter(s)	Hatching frequency, median hatching time, median survival of embryo/fry, deformation frequency in fry and hyperactivity in fry.	
3.4.8	Examination / Sampling	Every 24 h	
3.4.9	Monitoring of TS concentration	Yes	
3.4.10	Statistics	NOEC AND LOEC were determined for the test parameters and treatments.	

4 RESULTS

4.1	**Range finding test**	Range finding test was not performed.
4.1.1	Concentrations	Range finding test was not performed.
4.1.2	Number/ percentage of animals showing adverse effects	Range finding test was not performed.
4.1.3	Nature of adverse effects	Range finding test was not performed.
4.2	**Results test substance**	
4.2.1	Initial concentrations of test substance	0 (control), 0.1 mg/l, 0.3 mg/l, 0.6 mg/l, 1 mg/l, 3 mg/l, 6 mg/l, 10 mg/l, 30 mg/l and 60 mg/l.
4.2.2	Actual concentrations of test substance	The concentration of test substance was between 70-109% of the nominal concentration before renewal of test solutions. A mean value of 82% of the test substance was measured in the test solutions after 24 h of exposure which indicate that nominal concentrations can be used for further calculations.

4.2.3	Effect data	Please refer to table A7_4_3_2_1(01)-6	X
4.2.4	Concentration / response curve	Please refer to table A7_4_3_2_1(01)-6 for concentration/response relationship.	
4.2.5	Other effects	Significant hyperactivity in fry at 3 mg/l.	
4.3	**Results of controls**		
4.3.1	Number/ percentage of animals showing adverse effects	No control animal displayed adverse effects.	
4.3.2	Nature of adverse effects	No control animal displayed adverse effects.	
4.4	**Test with reference substance**	Not performed	
4.4.1	Concentrations	Not performed	
4.4.2	Results	Not performed	

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1	**Materials and methods**	Effects of the test substance on reproduction fitness were investigated using Zebra fish (*Danio rerio*) with hatching frequency, median hatching time, median survival of embryo/fry, deformation frequency in fry and hyperactivity in fry as endpoints.	
5.2	**Results and discussion**	Hatching time was significantly prolonged at 60 mg/l, median survival time was significantly lowered at 10 mg/l, the deformation frequency was significantly increased at 6 mg/l and significant hyperactivity was observed at 3 mg/l. No conclusive significant effects were observed for hatching frequency.	
5.2.1	NOEC	Hatching time: 30 mg/l Median survival time : 6 mg/l Deformation frequency : 3 mg/l Hyperactivity : 1 mg/l	X
5.2.2	LOEC	Hatching time : 60 mg/l Median survival time : 10 mg/l Deformation frequency : 6 mg/l Hyperactivity : 3 mg/l	X
5.3	**Conclusion**	The assessment of effects in Zebra fish (*Danio rerio*) was performed according to OECD guideline 212. The guideline criteria were fulfilled. The does-response relationships indicate that the test substance have on effects on Zebra fish embryo/fry from 3 mg/l.	
5.3.1	Other Conclusions	No other conclusions made.	
5.3.2	Reliability	Based on the study which in accordance to OECD guideline 212 the reliability indicator is 1.	
5.3.3	Deficiencies	No	

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	12/6/12
Materials and Methods	1.1 The report title is actually 'Ecotoxicological evaluation of medetomidine hydrochlorid – effects on embryo and larvae of sebrafish' although it is likely that the title provided is a proper reflection of the actual study undertaken. 2.1 Test conducted to Swedish standard SS 028193 which is comparable with OECD 212. 3.1.1 Batch number is reported as 1061171. 3.1.3 The purity of the active substance is not given in the report. 3.1.5 Details of solubility with temperature and pH is given in Doc IIIA Section 3. 3.1.6 The report just states that analysis of the test solutions to confirm nominal concentrations was undertaken at CRST laboratory in Finland. 3.4.1 The pH of the dilution water used in embryo/larvae test was stated to be 7.5 (7.3 to 7.6) and is reported as 7.03 to 7.65 in the actual test. 3.4.2 The fish stock used are stated as spawning stock. It is not specifically stated if the fish were fed or if there was any pre-treatment for disease. 3.4.4 The volume of the vessel is not specifically stated instead it is reported that 30 fertilised eggs were placed in 50 ml of test solution. When hatching was complete 5 to 10 larvae from each dish were transferred to a triplicate to strengthen the data for the probit analysis of mortality (see Table A7_4_3_2_1(01)—6)). 3.4.5 The pH is reported as 7.03 to 7.65 in the actual test (Appendix 2 of report).
Results and discussion	Applicant's version considered acceptable, noting the following: 4.2.3 table A7_4_3_2_1(01)-6 some amendments have been made to this table to ensure it ties in with Appendix 2 of the report e.g. information added on confidence limits, correction of number of deformations at 0.1 mg/L. 5.2.1/5.2.2 End points are based on nominal concentrations. NOEC and LOEC for hatching time: **no NOEC for hatching time is presented in the report.** The median time hatching days (Appendix 2) is shown in Table A7_4_3_2_1(01)—6 (this has been updated as per the report. The data appear to indicate that there is no real effects up to 6.0 mg/L in terms of hatching time. At 10 and 30 mg/ median hatching time is 4.1 and 4.3 days compared to the control at 3.6 days. No results are given for the 60 mg/L treatment (presumably due to mortality). The LOEC for hatching time is presented in the report as >60 mg/L (Table 2, but is indicated as 60 mg a.s./L) however the UKCA considers that it is difficult to say exactly what the LOEC is as it could be argued it is 10 mg/L as there does appear to be a slight delay in hatching time with the two highest concentrations. Due to the problems identified the UKCA considers a robust LOEC is difficult to derive. Hyperactivity: the report states the NOEC to be 1.0 mg /l and the LOEC as 3.0 mg /l (see Section 4.2 but no raw data presented). It should be noted that growth was not measured in this study. The report states the NOEC for deformations to be 1.0 mg/L (the percentage deformation in the three replicates were 6.7, 10 and 7.7%) and the LOEC 3.0 mg/L (the percentage deformation in the three replicates were 6.2, 20 and 13% compared with the control (values of 0, 0 and 6.7%)). It is also noted that this effect is concentration related and increases with concentrations. Details of what the deformation is are not provided. No details of effects on pigmentation are provided in this study. **Overall the lowest NOECs from this study are 1.0 mg /l for hyperactivity and deformation.**
Conclusion	Applicant's version considered acceptable
Reliability	3

Acceptability	Not acceptable
Remarks	This study was not undertaken to GLP and a quality assurance statement was not provided. Studies provided after 30 June 1988 should be conducted to GLP. The batch number for medetomidine hydrochloride is not given. Only summaries of the results are presented and so it is not possible to validate these to the raw data. The purity of the active substance used in this study is not provided in the report. It should be noted that no measurements were made of fish length as recommended by OECD 212. The lack of raw data and the fact that for some parameters it was considered that the derivation of robust NOECs and LOECs was problematic. Although some details are given of the analytical concentrations the method of analysis used for determining these is not given. Nor is a full report for the analysis presented.
	COMMENTS FROM ... *(specify)*
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A7_4_3_2_1(01)--1: Dilution water

Criteria	Details
Source	Swedish standard 028193
Salinity	No
Hardness	No information available
pH	7.3 (± 0.2)
Oxygen content	>95%
Conductance	<5 μScm^{-1}
Holding water different from dilution water	No

Table A7_4_3_2_1(01)--2: Test organisms

Criteria	Details
Species/strain	Zebra fish *Danio rerio*
Source	Singapore
Wild caught	No
Age/size	Egg/yolk sac fry
Kind of food	No information available
Amount of food	No information available
Feeding frequency	No information available
Post-hatch transfer time	No information available
Time to first feeding	No information available
Feeding of animals during test	No
Treatment for disease within 2 weeks preceding test	No

Table A7_4_3_2_1(01)--3: Test system egg/embryo development

Criteria	Details
Test type	Semistatic
Renewal of test solution	Test solution were renewed daily
Volume of test vessels	100 ml
Volume/animal	3.30 ml
Number of animals/vessel	30
Number of vessels/ concentration	2
Test performed in closed vessels due to significant volatility of TS	No

Table A7_4_3_2_1(01)--4: Test system embryo/fry development

Criteria	Details
Test type	Semistatic
Renewal of test solution	Test solution were renewed daily
Volume of test vessels	100 ml
Volume/animal	7.7 ml
Number of animals/vessel	13
Number of vessels/ concentration	3
Test performed in closed vessels due to significant volatility of TS	No

Table A7_4_3_2_1(01)--5: Test conditions egg/embryo and embryo/fry

Criteria	Details
Test temperature	25 ± 1° C
Dissolved oxygen	91-102
pH	7.5 (egg/embryo) and 7.0 ± 0.1 (embryo/fry)
Adjustment of pH	Yes
Aeration of dilution water	No
Intensity of irradiation	Away from daylight
Photoperiod	12 h

Table A7_4_3_2_1(01)--6: Results

Concentration (mg/l)	Egg/embryo mortality (24 h)	Hatching frequency	Median hatching time (days)	Survival (days)	Deformation frequency
0	5/30	100 100 100	3.6	13.9 13.3 14.0	6.7 0 0
0.1	4/30	100 100 100	3.7	14.0 13.0 13.7	0 0 0
0.3	3/30	100 100 100	3.9	13.9 14.4 14.3	6.7 0 0
0.6	0/30	100 100 100	3.7	14.1 14.3 13.7	0 11.1 6.2
1.0	6/30	100 100 100	3.6	13.2 14.0 14.1	7.7 10 6.7
3.0	1/30	100 100 100	3.5	13.2 14.2 13.6	13 20 6.2
6.0	4/30	100 100 100	3.9	13.6 11.9 13.4	21 20 20
10	3/30	100 100 100	4.1	11.8 11.5 12.3	33 25 31
30	0/20 0/30	100 100	4.3	5.4 7.2	40 52
60	0/20 0/30	100 70	-	5.0 5.3	44 50

Doc III A section 7.4.3.2.2 (01) Annex Point IIIA XIII 2.2

Sublethal effects on lumpfish (*Cyclopterus lumpus* L.) and Atlantic cod (*Gadus morhua* L.) larvae

			Official use only
		1 REFERENCE	
1.1	**Reference**	Bella J, Hilvarsson A, Granmo Å. (2005) Sublethal effects of a new antifouling candidate on lumpfish (*Cyclopterus lumpus* L.) and Atlantic cod (*Gadus morhua* L.) larvae. Biofouling **21**(3-4):207-216. (Published)	
1.2	**Reference**	Handa P, Fant C and Nyden M. (2006) Antifouling agent release from marine coatings – ion pair formation/dissolution for controlled release. Prog. Org. Coat. **57** (4):376-382. (Published)	X
1.3	**Data protection**	No	
1.3.1	Data owner	Public domain	
1.3.2	Criteria for data protection	No data protection claimed	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	No A7_4_3_2_2(01) serves as an addition to A7_4_3_2_1(01) performed according to guideline OECD TG 212.	
2.2	**GLP**	No Study performed according to scientific sound methods.	
2.3	**Deviations**	Yes Please refer to section 3.4	
		3 METHOD	
3.1	**Test material**	Medetomidine, as given in references.	
3.1.1	Lot/Batch number	S28	X
3.1.2	Specification	Please refer to reference 1.2 (Handa et al 2006).	X
3.1.3	Purity	> 99%	X
3.1.4	Composition of Product	Crystalline powder	
3.1.5	Further relevant properties	Please refer to doc IIIA section 2.	
3.1.6	Method of analysis	Test substance was analysed by NMR, please refer to reference 1.2 for details.	X
3.2	**Preparation of TS solution for poorly soluble or volatile test substances**	Test substance was dissolved in methanol for stock solutions. Test concentrations were obtained by diluting the stock solution in test media. The same amount of methanol was added to each experimental vessel. For details see table A7_4_3_2_2(01)-1.	X
3.3	**Reference substance**	No reference substance was used.	
3.3.1	Method of analysis for reference substance	No reference substance was used.	

Doc III A section 7.4.3.2.2 (01)
Annex Point IIIA XIII 2.2

Sublethal effects on lumpfish (*Cyclopterus lumpus* L.) and Atlantic cod (*Gadus morhua* L.) larvae

3.4 Testing procedure		
3.4.1 Dilution water	For details on dilution water see table A7_4_3_2_2(01)-2.	
3.4.2 Test organisms	For details on tested organisms see table A7_4_3_2_2(01)-3.	
3.4.3 Handling of embryos and larvae	Lumpfish: Fertilised eggs and the nests were guarded by males until hatching. Eggs were checked daily to establish exact day of hatching. Cod: Newly hatched larvae were brought aerated to the Sandgerði Marine Centre. For each test 12-24 h post-hatching larvae from one single female were used. Only active larvae were used for testing.	
3.4.4 Test system	For details on test type, renewal of TS solution, laboratory equipment, loading, replicates etc. see table A7_4_3_2_2(01)-4.	
3.4.5 Test conditions	For test conditions see table A7_4_3_2_2(01)-5.	X
3.4.6 Duration of the test	96 h exposure and 48 h recovery.	
3.4.7 Test parameter(s)	Respiration rate (lumpfish and cod) and skin colour adaptation (lumpfish).	
3.4.8 Examination / Sampling	Test parameters were recorded at 24, 48, 72 and 96 h of exposure and after 24 and 48 h in clean test media. Respiration rate: Following exposure to test item three lumpfish larvae or six cod larvae were transferred to 10 ml glass syringes containing 6 ml of fully oxygen saturated and thermo-regulated test media (10°C). After incubation, 3 h for lumpfish and 6 h for cod, 2 ml of the test media from each syringe were injected into a microrespiration chamber with an oxygen microelectrode and connected to an oxygen meter. A minimum of 7 blank samples (no larvae in syringe) were included to calibrate the larval oxygen consumption. Skin colour adaptation: Following exposure to test item larvae were transferred to a dark test vessel with a lid to avoid light disturbance. Dark, brown and light larvae were counted after 2 min. A typical skin colour for an unaffected larva would be dark.	
3.4.9 Monitoring of TS concentration	Yes Semistatic test system was sampled at start and after 12 h. Flow-through test system was sampled at 1, 6 and 12 h.	
3.4.10 Statistics	Differences between treatments were tested for significance using ANOVA. Dunnett's test was used to compare the control group to each treatment with significant differences to determine LOEC. Respiration data were log-transformed and colour adaptation data were arcsine-transformed to achieve normality prior to statistic calculations.	

4 RESULTS

4.1 Range finding test	No range finding test performed.	
4.1.1 Concentrations	No range finding test performed.	

Doc III A section 7.4.3.2.2 (01)
Annex Point IIIA XIII 2.2

Sublethal effects on lumpfish (*Cyclopterus lumpus* L.) and Atlantic cod (*Gadus morhua* L.) larvae

4.1.2	Number/ percentage of animals showing adverse effects	No range finding test performed.	
4.1.3	Nature of adverse effects	No range finding test performed.	
4.2	**Results test substance**		
4.2.1	Initial concentrations of test substance	Please refer to table A7_4_3_2_2(01)-6.	X
4.2.2	Actual concentrations of test substance	Analysed concentrations fluctuated between 70-130% (t=0) and 60-115% (t=12h) of nominal concentrations. Therefore, nominal concentrations were used for dose-response determinations and statistics.	X
4.2.3	Effect data	Respiration rate: Lumpfish larvae in the semistatic test system had a NOEC of 0.011mg/L and a LOEC of 0.013 mg/L after 96 h exposure. In the flow-through test system they had a NOEC of 0.001 mg/L and a LOEC of 0.005 mg/L after 96 h exposure. After 48 h recovery no significant differences could be observed between the larvae. No significant effects were observed for the cod larvae and therefore NOEC and LOEC could not be determined. For more details see reference 1.1 and table A7_4_3_2_2(01)-7.	X
		Skin colour adaptation: Lumpfish larvae in the semistatic test system had a LOEC of 0.013 mg/L after 96 h exposure caused by a decrease in the numbers of dark larvae and an increase in yellow larvae. NOEC was 0.003 mg/L. In the flow-through test system NOEC was 0.001 mg/L and LOEC 0.005 mg/L. After 24 h of recovery no differences were observed between exposed larvae and the controls. For more details see reference 1.1 and table A7_4_3_2_2(01)-8.	X
4.2.4	Concentration / response curve	Please refer to reference 1.1 (Bellas et al 2005).	
4.2.5	Other effects	No other effects observed.	
4.3	**Results of controls**		
4.3.1	Number/ percentage of animals showing adverse effects	No adverse effects were observed in controls or methanol controls.	
4.4	**Test with reference substance**	No reference substance was used.	
4.4.1	Concentrations	No reference substance was used.	
4.4.2	Results	No reference substance was used.	

Doc III A section 7.4.3.2.2 (01)
Annex Point IIIA XIII 2.2

Sublethal effects on lumpfish (*Cyclopterus lumpus* L.) and Atlantic cod (*Gadus morhua* L.) larvae

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1	**Materials and methods**	Sublethal effects of the test substance on juvenile lumpfish and cod were investigated. The test parameters were respiration rate and skin colour adaptation. The test organisms, 12-24 h old larvae, were exposed to the test substance during 96 h in a semistatic test system. Lumpfish larvae were also exposed in a flow-through test system for 96 h. During the exposure period larvae were sampled and used for measurements of respiration rate and skin colour adaptation. After exposure the recovery of the larvae were investigated.	
5.2	**Results and discussion**	The respiration rate for lumpfish was affected after 72 h of exposure with a LOEC between 0.001 and 0.002 mg/L. However, after 96 h of exposure the LOEC was 0.005 mg/L. The oxygen consumption rate shows a dose-response relationship between the test substance and respiration rate where the test substance seems to reduce the metabolic activity of exposed larvae after 72 h of exposure. After 48 h recovery no significant differences could be observed between exposed and unexposed lumpfish larvae. No effects were observed on the respiration rate of cod larvae. Cod larvae are less sensitive for impact on respiration rate by biocides which has been seen in other studies (reference 1.1).	X
		Skin colour adaptation was significantly affected after 96 h exposure in both test systems. The larvae in the flow-through test system were most sensitive with a NOEC of 0.001 mg/L and a LOEC of 0.005 mg/L. The semistatic test system had a NOEC of 0.003 mg/L and a LOEC of 0.013 mg/L. After 24 h recovery no significant differences could be observed between exposed and unexposed lumpfish larvae.	X
	5.2.1 NOEC	Respiration rate (96h): Lumpfish – 0.001mg/L Cod – $\geq$ 0.2 mg/L Skin colour adaptation (96h) : Lumpfish - 0.001 mg/L Cod- Not used in test	X
	5.2.2 LOEC	Respiration rate (96h): Lumpfish – 0.005 mg/L Cod – > 0.2 mg/L Skin colour adaptation (96h) : Lumpfish - 0.005 mg/L Cod – Not used in test	X
5.3		The methods used for assessing sublethal effects of the test substance in lumpfish and cod larvae are appropriate and have been described in scientific peer-reviewed literature. Effects on respiration rates and skin colour adaptation were determined after 24, 48, 72 and 96 h exposure. All controls displayed normal respiration rates and skin colour adaptation. The does-response relationships indicate that the test substance have sublethal effects on larvae from one fish specie.	
	5.3.1 Other Conclusions	No other conclusions made.	
	5.3.2 Reliability	Based on the methods which in accordance to acceptable scientific principles the reliability indicator is 2.	X

Doc III A section 7.4.3.2.2 (01)
Annex Point IIIA XIII 2.2

Sublethal effects on lumpfish (*Cyclopterus lumpus* L.) and Atlantic cod (*Gadus morhua* L.) larvae

5.3.3 Deficiencies	Yes The analysed concentrations of test substance were not used for dose-response determination. The fluctuations were ± 30% at t=0.	X

Evaluation by Competent Authorities

Use separate "evaluation boxes" to provide transparency as to the comments and views submitted

EVALUATION BY RAPPORTEUR MEMBER STATE

Date 11/8/10

Materials and Methods

Applicant's version considered acceptable, noting the following

1.2 There is no cross reference in report reference 1.1 to the Handa report.

3.1.1 A batch number is not given in the report.

3.1.2 There is no cross reference in the report to the Handa reference and therefore it cannot be validated that the active substance was produced in this way.

3.1.3 No purity is given in the report.

3.1.6 The report refers to analysis by gas chromatography-mass spectrometry.

3.2 The report states that <100 µl/l of methanol was used.

3.4.5 The concentrations in the study are actually presented in terms of nM. The concentrations tested are as per Table 1 and where comments are available in the text linking these to mg/l concentrations these are presented in Table 2.

Table 1. Concentrations of medetomidine used in the toxicity tests.

Test	Species	Medetomidine (nM)
Semistatic	Lumpfish	4 6 10 16 18 30 54 64 90 162 256 270 486 810 1024
	Cod	4 16 64 256 1024
Flow-through	Lumpfish	5 25 125 625

Table 2 Comments in the text provide the following mg/l concentrations of medetomidine (where concentration conversions are not given the nM concentration is not included)

Test	Species	Units	Medetomidine concentration					
Semi static	Lump fish	nM	4	10	30	64	256	486
		µg/l	0.8	2	6	13	51	96
Flow through	Lump fish	nM	5	25				
		µg/l	1	5				

Using this information the UKCA has calculated the concentrations in terms of µg/L for all the concentrations used. These are shown in Table 3 below:

Table 3 Calculated values for the concentrations in terms of nM and µg/L

Lump fish and cod concentrations (semi-static)				Lump fish concentrations (flow through)	
nM	µg/L	nM	µg/L	nM	µg/L
4	0.8	162	32.4	5	1
6	1.2	256	51.2	25	5
10	2	270	54	125	25

16	3.2	486	97.2	625	125
18	3.6	810	162		
30	6	1024	204.8		
90	18				

Results and discussion

4.2.1 See comment at 3.4.5 above. The concentrations presented in Table A7_4_3_2_2(01)-6 do not tie in with those presented in the report (see Table 3 above).

4.2.2 Usually in OECD tests results are only based on nominal concentrations if the analysis shows concentrations to be within ±20% of nominal concentrations. Where this is not the case then mean measured concentrations should be used.

4.2.2 Note that the concentration is not always referred to consistently. On report p 210 it refers to a concentration as 480 nM however table 1 of concentrations only contains a concentration of 486 nM.

4.2.3 The following amendments are noted: It is important to note that no NOECs are actually given in the report, rather these have to be interpreted from the LOECs and information presented. It is important to note that the UKCA also highlights any trends in the data, even when these are not statistically significant and refers to NOECS or LOECs based on these. To enable this approach to be clearly understood full details are presented for each parameter measured. Also it should be noted that some of the LOECs cited here are actually presented in the report (so are therefore statistically derived).

Respiration rate: flow through : lump fish. The LOEC given for respiration in the report is 5nM (1 µg/l i.e. 0.001 mg /l) at 72 h and 25 nM (5 µg/l i.e. 0.005 mg/l) at 96 h (p210 of report)). **At 72 hours this is the lowest concentration tested and so a NOEC at 72 h cannot be derived. However it is noted at 48 hours there was no significant decrease in respiration.**

Respiration rate: semi static: lump fish. There was no effect after 24 h exposure but the reported LOEC after 48 h was 480 nm (96 µg/l) and after 72 h was 10 nM (2 µg/l), after 96 h effects were more pronounced but there was only a significant difference at 60 nM (12 µg/l).

Overall for lump fish the lowest respiration LOEC is 0.001 mg after 72 h in the flow through experiment since a lower concentration was not tested a NOEC cannot be derived at 72 h. In the semi-static experiment the LOEC was 2 µg/l and **although a 72 hour NOEC is not actually presented it is considered that the lowest NOEC was 6nM (concentration not given in mg/l but approximately 1.2 µg/l as 10 nM is stated to be equal to 2 µg/l plus see Table 3) due to effects observed at 10nM (2 µg/L).** This is simply on the basis of the LOEC stated in the report (taking the NOEC as the concentration below this).

Cod: the RMS agrees that there were no effects on the respiration of cod.

Recovery period respiration: The statement on 48 h recovery and no significant differences is further clarified in the report as no significant differences in respiration rate were found between larvae pre-exposed to 625 nM (125 µg/l) and control larvae in the flow through experiments or between 1024 nM (205 µg/l) exposed larvae and the control in semi-static conditions. The respiration rate recovered from 45% inhibition in pre-exposed larvae to 18% after the recovery period.

Skin colour adaptation, flow through: **The 96 hour LOEC in the report is given as 30 nM (6 µg/l) and hence the NOEC (although not actually presented in the report but from taking the concentration below the reported LOEC) is 5nM (0.001 mg/l).** It is noted however that some of these concentrations do not tie in with those stated to have been tested in Table 1 of the report e.g. 30nM is not presented a test concentration.

Skin adaptation semi static: A NOEC for this is not specifically stated in the report. It is stated that after 24 hours exposure to 4 nM (0.8 µg/l) there is a decrease in the number of dark larvae compared to the control with no dark larvae found at 256 nM (51 µg/l). The effect is more obvious with increasing exposure time and after 72 hours there were no dark larvae found at (64 nM) 13 µg/l. A statistically derived LOEC is not presented in the report but in view of this effect the UKCA considers it may actually be 0.8 µg/l. This is also illustrated in Figure 6 of the report where by visual observation of the figures only it appears that in the control there are about 70% dark larvae and only approximately 40% in the 4 nM treatment. This difference is significant but not significant at the next

concentration of 16 nM but it is also significant at 64 nM. The results are summarised in Figure 6 below taken from the report. There is a clear effect at 64 nM (13 µg/l) whilst at 4nM (0.8 µg/l) and 16nM (concentration only presented in the report in nM) the significance varies at different time points in the experiment. The results are shown in Figure 6; at 24 hours there is a significant reduction in dark larvae, 48 hours a significant increase in yellow larvae, 72 hours differences not significant, 96 hours a significant decrease in brown larvae. Some effects were also seen at 16 nM at 48 and 72 hours (see Figure 6). **Overall the RMS is of the view that it is not possible to derive a reliable, robust NOEC in this study under semi-static conditions for skin adaptation due to effects at the lowest concentration tested (4 nM; 0.8 µg/l) and indications that there is a trend with increasing dose.**

Recovery period adaptation: the report states that the percentage of dark and brown larvae increased from 5.4% at 64 nm (13 µg/l) during 96 h exposure to 82% (with respect to the control) after 24 h recovery. The RMS notes however that the number of dark larvae is still below the control (see Figure 6).

Finally the RMS notes that the values presented in the tables referred to Table A7_4_3_2_2(01)-7: Respiration rate, Table A7_4_3_2_2(01)-8: Skin colour adaptation) are not actually presented in the report and the raw data are not available either. Instead the RMS has used comments derived from the report and the figures in the report to derive the comments listed above.

To enable the retention of the standard document structure figure 6 from the report is reproduced below rather than inserted here.

Conclusion	Applicant's version considered acceptable, noting the following: 5.2 See comments at 4.2.3. 5.2.1 It should be noted that the UKCA has highlighted where there are potential trends in the data even when these are below the statistically derived LOEC. NOEC respiration rate (flow through): lump fish: the LOEC at 72 h was 5nM (1 µg/l) this was the lowest concentration tested. In view of this it is considered that overall the NOEC at 72 hours for the study is < 1 µg/l. Respiration rate in the semi-static study for lump fish: the LOEC after 48 h was 480 nM (96 µg/l) and after 72 h was 10 nM (2 µg/l). No effects on respiration were seen for cod. NOEC colour adaptation lump fish semi-static: the data did not result in a statistically derived LOEC being presented in the report. However, the RMS did not consider overall that a robust NOEC could be derived from the information presented due to effects seen at the lowest concentration tested (0.8 µg/l) in the semi-static study. The UKCA therefore considers the NOEC is < 0.8 µg/l. It was considered important to note that effects were seen at the very low concentrations tested. In the flow through study a LOEC of 30 nM (6µg/l) was reported. The NOEC is therefore considered to be 5 nM (1 µg/l). But it is noted that there were some issues with the concentrations reported in the results not tying in with those stated to be tested e.g. at 30nM. 5.2.2 LOEC respiration rate flow through: lump fish the report states that the LOEC is 5 nM (1µg/l) at 72 h and 25 nM (5 µg/l). LOEC respiration semi-static the LOEC at 48 h was 480 nM (96 µg/l) and after 72 h was 10 nM (2µg/l). Colour adaptation: the 96 h LOEC presented in the report was 30 nM (6 µg/l). The data did not result in a statistically derived LOEC for the semi-static study but comments on effects seen are discussed in the NOEC section above. Effects were observed at 4 nM (0.8 µg/l) and therefore the UKCA considers that this constitutes the LOEC.
Reliability	4
Acceptability	Not acceptable
Remarks	The purity, batch number and manufacture of the medetomidine used in this study could not be confirmed since there was no cross reference in the report to the Handa reference citing the method of production. The measured concentrations in the study varied between 70-130% but nevertheless the end points derived were based on nominal concentrations. For OECD studies it is usual to use mean measured concentrations where the fluctuations are ±20% if this approach is also applied to this study the end points given may be an overestimate. The full analytical results report is not presented to allow verification of the concentrations. Additionally tables of NOECs were not presented in the report instead it was necessary to try and derive these from verbal comments in the reports and the use of the figures. LOECs were presented. There were some variations between the concentrations results were reported for and those which were stated to have been tested. The raw data were not available either to enable clarification of these issues. Overall these limitations made the interpretation of this interesting published paper difficult and it was concluded that the results should be treated with caution.

	COMMENTS FROM ... *(specify)*
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub) heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*

Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Figure 6 from the report for colour adaptation is inserted below (full details are given in the title below the tables).

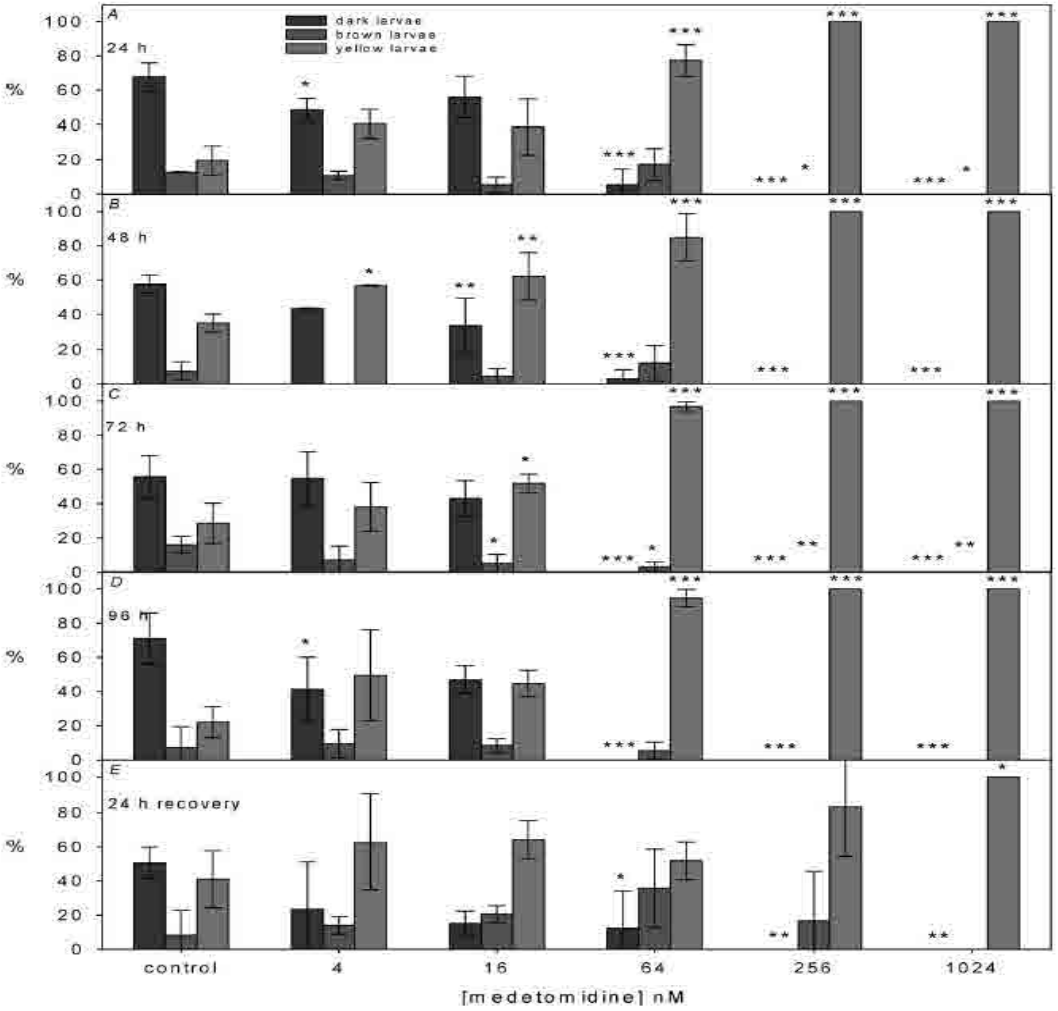


Figure 6. Colour adaptation of lumpfish larvae after 24 h (A), 48 h (B), 72 h (C) and 96 h (D) exposure to different medetomidine concentrations, and after 24 h recovery in clean seawater (E) in semistatic experiments. Error bars = the SD for each treatment ($n = 3$). *significant differences at $p < 0.05$; **significant differences at $p < 0.01$; ***significant differences at $p < 0.001$.

Table A7_4_3_2_2(01)-1: Preparation of TS solution for poorly soluble or volatile test substances

Criteria	Details
Dispersion	No
Vehicle	Methanol (p.a)
Concentration of vehicle	100 µl/l
Vehicle control performed	Yes
Other procedures	No other procedures

Table A7_4_3_2_2(01)-2:Dilution water

Criteria	Details
Source	Natural sea water from 50 m depth in the earth, Sandgerði, Iceland
Salinity	32-33 ppt
Hardness	Not measured
pH	Not measured
Oxygen content	Not measured
Conductance	Not measured
Holding water different from dilution water	No

Table A7_4_3_2_2(01)-3:Test organisms

Criteria	Details
Species/strain	Lumpfish (*Cyclopterus lumpus*) and Atlantic cod (*Gadus morhua*)
Source	Sandgerði, Iceland
Wild caught	Yes: Lumpfish were caught 1 km west of Sandgerði, south west Iceland No: Atlantic cod were obtained from Grindavik aquaculture experimental station
Age/size	Newly hatched larvae were used tests (12-24 h post-hatch)
Kind of food	No information available for parental fish. Larvae used for tests were living on yolk sack.
Amount of food	No information available for parental fish. Larvae used for tests were living on yolk sack.
Feeding frequency	No information available for parental fish. Larvae used for tests were living on yolk sack.
Post-hatch transfer time	No information available.
Time to first feeding	No information available for parental fish. Larvae used for tests were living on yolk sack.
Feeding of animals during test	No
Treatment for disease within 2 weeks preceeding test	No

Table A7_4_3_2_2(01)-4:Test system

Criteria	Details
Test type	Semistatic with lumpfish larvae and cod larvae Flow-through with lumpfish larvae
Renewal of test solution	Semistatic test system: media were renewed every 12 h. Flow-through test system: water flow-rate 100 ml/min, test substance 2 ml/h
Volume of test vessels	400 ml
Volume/animal	Semistatic test system: Lumpfish 13 ml/animal, Cod 8 ml/animal Flow-through test system: 13 ml/animal (lumpfish)
Number of animals/vessel	Semistatic test system: Lumpfish 25-30, Cod 45-50 Flow-through test system; 25-30 (lumpfish)
Number of vessels/ concentration	Both test systems: 3 vessels per concentration including control and methanol control
Test performed in closed vessels due to significant volatility of TS	No

Table A7_4_3_2_2(01)-5:Test conditions

Criteria	Details
Test temperature	10 ± 0.5°C
Dissolved oxygen	Not measured
pH	Not measured
Adjustment of pH	No
Aeration of dilution water	No
Intensity of irradiation	Not measured
Photoperiod	No information available

Table A7_4_3_2_2(01)-6: Test concentrations (nominal); see amendments in text above.

Test	Test item (mg/L)
Semistatic	
Lumpfish	0.0008 0.0012 0.0020 0.0032 0.0036 0.0060 0.0108 0.0128 0.0180 0.0324 0.0513 0.0541 0.0973 0.1622 0.2051
Cod	0.0008 0.0032 0.0128 0.0513 0.2051
Flow-through	
Lumpfish	0.0010 0.0050 0.0250 0.1252

Table A7_4_3_2_2(01)-7: Respiration rate (see amendments above)

Test species and system	Exposure time (h)	NOEC (mg/L)	LOEC (mg/L)
Lumpfish, semistatic	24	No effects	No effects
	48	0.054	0.098
	72	0.0012	0.002
	96	0.011	0.013
Lumpfish, flow-through	24	No effects	No effects
	48	No effects	No effects
	72	No effects	0.001
	96	0.001	0.005
Cod, semistatic	24	No effects	No effects
	48	No effects	No effects
	72	No effects	No effects
	96	No effects	No effects

Table A7_4_3_2_2(01)-8: Skin colour adaptation (see amendments above)

Test species and system	Exposure time (h)	NOEC (mg/L)	LOEC (mg/L)
Lumpfish (semistatic)	24	0.003	0.013
	48	Effect at lowest concentration used	0.0008
	72	0.0008	0.003
	96	0.003	0.013
Lumpfish (flow-through)	24	No effects	No effects
	48	No effects	No effects
	72	No effects	No effects
	96	0.001	0.005

Doc III A section 7.4.3.2.3 (01)
Annex Point IIIA XIII 2.2

Determination of the effects on the early-life stage of the Sheepshead minnow *(Cyprinodon variegates)*

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted] Medetomidine: Determination of the effects on the early-life stage of the Sheepehead minnow *(Cyprinodon variegates).* [redacted] (Unpublished)	X
1.2	**Data protection**	Yes	
1.2.1	Data owner	I-Tech AB	
1.2.2	Criteria for data protection	Data on new a.s for first approval /authorisation	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes OECD 210.	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 METHOD	
3.1	**Test material**	Medetomidine	
3.1.1	Lot/Batch number	MED/FP/1540/42	
3.1.2	Specification	AS given in section 2	
3.1.3	Purity	**XXXXX** %	
3.1.4	Composition of Product	Crystalline powder.	
3.1.5	Further relevant properties	Molecular weight: 200.28 g/mol Water solubility: 0.19 g/L at 20°C	X
3.1.6	Method of analysis	Liquid chromatography, electron ionasation and tandem mass spectrometry. Please refer to IVA7.4.3.2.3 for details.	
3.2	**Preparation of TS solution for poorly soluble or volatile test substances**	Not performed	
3.3	**Reference substance**	No reference substance used.	
3.3.1	Method of analysis for reference substance	No reference substance used.	

3.4	**Testing procedure**		
3.4.1	Dilution water	Salinity adjusted natural sweater. Details on dilution water see table A7_4_3_2_3-1.	X
3.4.2	Test organisms	Sheepshead minnow, *Cyprinodon variegates*. For further details on test organism see table A7_4_3_2_3-2.	
3.4.3	Handling of embryos and larvae	Handling of embryos and larvae were performed according to OECD guideline 210.	
3.4.4	Test system	Flow-through test system, for further details see table A7_4_3_2_3-3.	
3.4.5	Test conditions	Details on test conditions see table A7_4_3_2_3-4.	X
3.4.6	Duration of the test	28 days post hatch	
3.4.7	Test parameter(s)	Hatching frequency, survival, length and weight.	
3.4.8	Examination / Sampling	Daily	
3.4.9	Monitoring of TS concentration	Yes	
3.4.10	Statistics	Hatchability and survival data, survival, standard length and dry weight were analysed using Minitab to determine statistical differences between and within treatments. Hatchability and survival data replicates were pooled and Fisher's Exact test was used to determine significant differences between hatchability and survival in the control and treatments. Length data and weight data replicates were pooled and analysed with Kruskal-Wallis test followed by a series of Mann-Whitney tests to determine differences between the control and each treatment in length and weight. NOEC and LOEC values were based on the results from the statistical analyses.	
		4 RESULTS	
4.1	**Range finding test**	Range finding test was performed but not presented in study report for the full study.	
4.1.1	Concentrations	10, 100 and 1000 µg/l	X
4.1.2	Number/ percentage of animals showing adverse effects	Significant effects in all concentrations.	
4.1.3	Nature of adverse effects	Weight development decrease compared to control animals.	
4.2	**Results test substance**		
4.2.1	Initial concentrations of test substance	0, 1.0, 3.2, 10, 32, 100 and 320 µg/l.	
4.2.2	Actual concentrations of test substance	The mean measured concentration of test substance was between 86-130% of the nominal concentration. In a majority of the measurements the concentration was within the 80-120% and therefore nominal concentrations were used reporting the results.	X

4.2.3	Effect data	Please refer to table A7_4_3_2_3-5	X
4.2.4	Concentration / response curve	Please refer to table A7_4_3_2_3-5 for concentration/response relationship and the document IV_A7_4_3_2_3.	
4.2.5	Other effects	Paler coloration of the larvae in the test concentrations 10 – 320 µg/l compared to the control was observed. 5 fish from each test concentration were therefore photographed at the end of the study. For further information please refer to document IV_ A7_4_3_2_3. Due to the qualitative nature of this endpoint the effect was not statistically analysed.	X
4.3	**Results of controls**		
4.3.1	Number/ percentage of animals showing adverse effects	No control animal displayed adverse effects.	
4.3.2	Nature of adverse effects	No control animal displayed adverse effects.	
4.4	**Test with reference substance**	Not performed	
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	Effects of medetomidine on the early-life stage of the Sheepshead minnow (*Cyprinodon variegates*) was investigated according to OECD guideline 210. Hatching, survival, dry weight and standard length was measured on fry exposed for 28 days post-hatch.	
5.2	**Results and discussion**	No effects were seen on hatchability or larval survival while a decrease in larval standard length and dry weight were observed in the exposed animals compared to the controls. It was also observed that larvae in test concentrations 10 – 320 µg/l were paler in coloration to those in the control treatment. This endpoint was however not statistically analysed due to its qualitative nature.	X
5.2.1	NOEC	Hatchability : 320 µg/l Survival : 320 µg/l Length : 32 µg/l Dry weight : 1.0 µg/l	
5.2.2	LOEC	Hatchability : >320 µg/l Survival : >320 µg/l Length : 100 µg/l Dry weight : 3.2 µg/l	
5.3	**Conclusion**	The assessment of early-life stage effects on Sheepshead minnow (*Cyprinodon variegates*) was performed according to OECD guideline 210. The dose-response relationships indicate that the test substance has effects on Sheepshead minnow (*Cyprinodon variegates*) from 3.2 µg/l and that weight development is the most sensitive parameter.	
5.3.1	Other Conclusions	No other conclusions.	
5.3.2	Reliability	Based on the study which in accordance to OECD guideline 210 the reliability indicator is 1.	
5.3.3	Deficiencies	No	

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	10/1/13
Materials and Methods	Applicant's version considered acceptable, noting the following: 1.1 The fish species is the 'Sheepshead minnow' and although the report refers to the species as 'Cyprinodon variegates' the RMS considers that this species is more usually called 'Cyprinodon variegatus'. 3.1.5 Details of solubility with temperature and pH is given in Doc IIIA Section 3. 3.4.1 The dilution water was natural sea water (not natural sweater). 3.4.5 Dissolved oxygen levels were stated to be 6.2 to 7.4 mg/L (87 – 103%); Section 5.5. The pH was 8.1 to 8.3.
Results and discussion	Applicant's version considered acceptable, noting the following: 4.1.1 The test concentrations in the range finding study are not presented in this report. 4.2.2 The mean percentage nominal concentration was 105-120% and results were based on nominal concentrations. It is noted in Table A7_4_3_2_3-5 that the third column is for 'survival' and the results presented are for the two individual replicates. 5.2 Statistical analysis showed there were no significant differences between the length of fry in the control and the 1.0, 3.2, 10 and 32 µg/L exposure treatments. A significant difference was observed between the control and the 100 and 320 µg/L exposure treatments. For larval weight analysis showed that there was a significant difference between the control and exposure treatments of 3.2 µg/L and above. 5.2.5 A statistically derived NOEC for fish pigmentation was not provide however the UKCA considers the NOEC to be 3.2 µg a.s./l based on effects at 10 µg a.s./l. 5.3.2 Observations on the start and end of hatching and the numbers of larvae hatching each day were not presented. This is listed under observations in OECD 210 for which some or all of the data may be available and is not a specific validity criteria. The study validity criteria were met.
Conclusion	Applicant's version considered acceptable.
Reliability	1
Acceptability	Acceptable
Remarks	None.
	COMMENTS FROM ... *(specify)*
Date	*Give date of comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table A7_4_3_2_3-1: Dilution water

Criteria	Details
Source	Natural seawater and dechlorinated fresh water
Salinity	25‰ (± 2‰)
Hardness (fresh water supply)	30.3-41.7 mg/l
pH	8.1-8.3
Oxygen content	87 – 103 %
Conductance	189 - 263 µScm^{-1}
Holding water different from dilution water	No

Table A7_4_3_2_3-2: Test organisms

Criteria	Details
Species/strain	Sheepshead minnow *Cyprinodon variegates*
Source	[redacted]
Wild caught	No
Age/size	Less than 24 h old eggs in morula or grastula stage of development.
Kind of food	Hatch day – post hatch day 10: live *Artemia salina* Post hatch day 11 – end of study: live *Artemia salina* and pellet food
Amount of food	Hatch day – post hatch day 10: 2.0 ml/fry/feed Post hatch day 11 – end of study: 3.0 ml/fry/feed and pellet food *ad libitum*
Feeding frequency	Twice per day
Post-hatch transfer time	>24 h
Time to first feeding	First day post hatch
Feeding of animals during test	First day post hatch until end of study
Treatment for disease within 2 weeks preceding test	No

Table A7_4_3_2_3-3: Test system

Criteria	Details
Test type	Flow-through
Renewal of test solution	7.5 times per day
Volume of test vessels	Working volume 9.5 L
Volume/animal	3.2 embryos / L
Number of animals/vessel	15 per incubation cup
Number of vessels/ concentration	2 with 2 egg cups within each vessel
Test performed in closed vessels due to significant volatility of TS	No

Table A7_4_3_2_3-4: Test conditions

Criteria	Details
Test temperature	25 ± 1° C
Dissolved oxygen	91-102
pH	8.8-8.3
Adjustment of pH	Yes
Aeration of dilution water	No
Intensity of irradiation	562 (average)
Photoperiod	16 h

Table A7_4_3_2_3-5: Results

Concentration (µg/l)	Hatching frequency (pooled)	Suvival	Mean length (mm) (pooled)	Mean weight (mg dw) (pooled)
0	95%	100% 100%	18.95	48.9
1.0	98%	100% 97%	19.6	49.3
3.2	97%	100% 97%	18.95	43.7
10	95%	97% 100%	18.74	43.4
32	93%	100% 100%	18.24	39.0
100	95%	100% 100%	17.43	33.2
320	98%	97% 100%	15.43	23.1

Doc III A section 7.4.3.2.4 (01)
Annex Point IIIA XIII 2.2

Effects on melanophore formation and aggregation

			Official use only
		1 REFERENCE	
1.1	**Reference**	[redacted] Medetomidine: Development and assessment of an assay for determining effects on zebra fish melanophore formation and aggregation. [redacted] (Unpublished)	
1.2	**Data protection**	Yes	
1.2.1	Data owner	I-Tech AB	
1.2.2	Criteria for data protection	Data on new a.s for first approval /authorisation	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	No. Study performed to develop and assess an assay.	
2.2	**GLP**	No	
2.3	**Deviations**	No	
		3 METHOD	
3.1	**Test material**	Medetomidine	
3.1.1	Lot/Batch number	MED/FP/1540/42	
3.1.2	Specification	AS given in section 2	
3.1.3	Purity	[redacted] %	
3.1.4	Composition of Product	Crystalline powder.	
3.1.5	Further relevant properties	Molecular weight: 200.28 g/mol Water solubility: 0.19 g/L at 20°C	X
3.1.6	Method of analysis	Liquid chromatography and electrospray ionisation-tandem mass spectrometry. Please refer to IVA7.4.3.2.4 Appendix 1 for details.	
3.2	**Preparation of TS solution for poorly soluble or volatile test substances**	Not performed	
3.3	**Reference substance**	1-phenyl 2-thiourea (PTU) that inhibit melanogenesis as negative control. α-melanocyte stimulation hormone (MSH) that stimulate melanogenesis as positive control.	
3.3.1	Method of analysis for reference substance	Liquid chromatography and electrospray ionisation-tandem mass spectrometry. Please refer to IVA7.4.3.2.4 Appendix 1 for details.	

3.4	**Testing procedure**		
3.4.1	Dilution water	Filtered and de-chlorinated tap water with salts added to maintain hardness levels. UV-sterilised and filtered to 10 µm. The water was delivered to temperature controlled header tank and re-filtered to 1µm before use.	
3.4.2	Test organisms	Zebra fish (*Danio rerio*)	
3.4.3	Handling of embryos and larvae	Embryos used were obtained from brood stock held at Brixham Environmental Laboratory. The adult fish were reared on site. Pairs of adult sexually mature fish were transferred to breeding conditions on the day previous to the initiation of the test. Embryos produced were checked for viability, cleaned to prevent fungal infection and transferred to test conditions.	
3.4.4	Test system	Test vessels were 250 ml glass beakers with 200 ml of test solution maintained on a black background to create uniform test conditions. Duplicate replicates were used for medetomidine and negative control PSU, each replicate contained 10 embryos. Positive control MSH was run in triplicates with 2 embryos per replicate in microplates.	
3.4.5	Test conditions	Test temperature was 28±2° C with a photoperiod of 14:10 (light:dark).	
3.4.6	Duration of the test	96 h	
3.4.7	Test parameter(s)	Number of melanophores, melanophore aggregation, surface area of melanophores, total area measured and percentage coverage by melanophores.	
3.4.8	Examination / Sampling	Developing embryos/larvae were examined daily for mortality and other abnormalities. At the end of exposure surviving embryos were transferred to a petri-dish and individually photographed.	
3.4.9	TS concentration	39, 390 and 6600 µg/l (measured value)	
3.4.10	Reference substance concentration	60 and 75 µM 1-phenyl 2-thiourea (PTU) 1 mM α-melanocyte stimulation hormone (MSH)	X
3.4.11	Monitoring of TS concentration	Yes at 24 and 96 h.	
3.4.12	Statistics	Power analysis was undertaken on all endpoints in order to assess which might be most sensitive for further use and to assess the number of animals required for a full study.	

4 RESULTS

4.1	**Range finding test**	Not performed.	
4.2	**Results test substance**		
4.2.1	Nominal concentrations of test substance	5.6, 56 and 560 µg/l	
4.2.2	Actual concentrations of test substance	39, 390 and 6600 µg/l. Due to a dilution error of the stock solutions the measured test concentration was approximately and order of magnitude higher than intended.	

4.2.3	Effect data	Survival was not affected by medetomidine or PTU. MSH, the positive control was highly toxic to the embryos giving 100% mortality after 96 h. Melanophore development and aggregation was not affected by medetomidine when compared visually to control organisms. PTU appears to severely retard melanophore development but not aggregation.	X
4.2.4	Concentration / response curve	Please refer to figures 1-5 in document IV_ A7_4_3_2_4 for photographic results.	
4.2.5	Other effects	The results for all endpoints indicate a high variability. Power analysis indicates that in a study of 6 treatments, in order to indentify a 10% reduction in melanophores approximately 250 organisms would be required per treatment. For the least variable endpoint, coverage, approximately 110 organisms would be required to detect a 10% difference.	X

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1	**Materials and methods**	Effects of medetomidine on melanophore development and aggregation were investigated using Zebrafish (*Danio rerio*). Number of melanophores, melanophore aggregation, surface area of melanophores, total area measured and percentage coverage by melanophores were examined in embryo/larvae exposed for 4 days post-hatch.
5.2	**Results and discussion**	
5.2.1	NOEC	Not determined
5.2.2	LOEC	Not determined
5.3	**Conclusion**	Melanophore development and aggregation in Zebrafish can be impacted by the presence of chemicals, for example PTU, but medetomidine did not produce a sufficient response to be able to demonstrate an effect when compared to control organisms. The study also demonstrates that melanophore development and aggregation in this species is highly variable. The impact of that is that the number of organism required for a sensitive assay is excessively high.
5.3.1	Other Conclusions	No other conclusions.
5.3.2	Reliability	2 due to the lack of GLP.
5.3.3	Deficiencies	No