C.2.8. Fish, Early-Life Stage (FELS) Toxicity Test (OECD TG 210)

Status: Assay validated by the OECD.

390. Modality detected/endpoints: This test has no endocrine-specific endpoints. However, there is limited evidence to suggest that some thyroid system disrupters are able to interfere with metamorphosis of the fish embryo to the larva.

Background to the assay

391. This test is widely used as a sub-chronic assay for non-endocrine disrupting (ED) chemicals, and can be used to predict concentrations causing chronic effects on growth and reproduction in fish. It was developed before concerns about endocrine disrupting chemicals (EDCs) arose and cannot be used to identify these chemicals. It exposes fish from immediately post-fertilisation to the larval free-feeding stage (28-60 days post-hatch [dph], depending on species). Permitted species include rainbow trout (*Oncorhynchus mykiss*), fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), sheepshead minnow (*Cyprinodon variegatus*) and silverside (*Menidia* sp.). The main endpoints include mortality, time to hatching, hatching success, growth, morphological abnormalities and abnormal behaviour.

392. Although the test does not have endpoints that specifically respond to EDCs alone, there are limited data which show that it is responsive to certain thyroid-disrupting chemicals. It is known that thyroid hormone receptors TRα and TRβ are both present in fish early embryos and larvae (Power et al., 2001), and that maternally derived thyroxine (T4) is important for thyroid-dependent processes in fish early life stages (Nelson et al., 2014). One of these processes is swimbladder inflation, an endpoint which can be recorded in the FELS test, and which is vital for the survival of fish fry. It has been shown, for example, that fathead minnow embryos exposed to a thyroid peroxidase (TPO) inhibitor (2-mercaptobenzothiazole) do not develop inflated swimbladders, probably because inhibition of TPO leads to decreased thyroid hormone synthesis (Villeneuve et al., 2013; Nelson et al., 2014). Also, Liu and Chan (2002) have shown that metamorphosis from embryo to larva in zebrafish is arrested by exposure to amiodarone (a TR antagonist) and by the goitrogen methimazole. Furthermore, Shi et al. (2008) demonstrated that the thyroid disrupter perfluorooctanesulfonic acid (PFOS) is able to delay hatching and cause developmental malformations in zebrafish embryos while upregulating two thyroid-related developmental genes, *hhex* and *pax8*. However, it is important to note that many non-ED chemicals will also cause these types of apical response, but by different mechanisms.

When/why the assay may be used

393. For an existing chemical, it is quite likely that data from a FELS (OECD TG 210) will already be available. If this is the case, indications of damage to the metamorphosis of fish embryos to larvae could be used as supporting data for a case that the chemical may be a thyroid disrupter. However, as stated above, there are many non-EDCs which are also

able to damage fish metamorphosis. Given the limited data (with respect to endocrine disruption) available from a FELS test, it would generally not be appropriate to request this assay especially to evaluate a suspected thyroid-acting ED.

394. Caution should be used when negative results are obtained with certain types of chemicals because absorption into the embryo via the chorion may have been impeded. Development of the OECD Fish Embryo Acute Toxicity (FET) Test (OECD TG 236) with zebrafish showed that this applies in particular to chemicals with a molecular weight ≥3kDa and a very bulky molecular structure. Absorption of these chemicals will take place at a higher rate after hatching, but delayed hatch may therefore also protect the embryo from other forms of toxicity. Although it is known that fish embryos have some metabolic capacity (e.g. Weigt et al. [2011]), this may be less efficient than in juveniles and adults, so use of the test with EDCs that require metabolic activation may give false negatives.

395. In order to provide information relevant for assessing whether or not a chemical may fulfil the WHO/IPCS (2002) definition of an ED, the study design has to be sufficiently robust to demonstrate the presence or absence of effects. In the dose selection, the investigator should also consider and ensure that data generated are adequate to fulfil the regulatory requirement across OECD countries as appropriate (e.g. hazard and risk assessment and labelling, ED assessment, etc.). The top dose or concentration should be sufficiently high to give clear systemic (i.e. non endocrine-specific) toxicity in order to ensure that a wide range of exposures (high to low) is tested. However, endocrine effects observed solely in the presence of clear systemic toxicity should be interpreted with caution and may be disregarded when sufficiently justified to be caused by secondary effects which are unlikely to be due to endocrine activity. The reason for this advice is a concern that some endocrine active substance (EAS) sensitive assays are being run at doses/concentrations of EASs that are too low to trigger direct impacts on the endocrine system. This guidance document is not the place to address this issue directly, but it should be considered when EAS-sensitive test guidelines (TGs) are revised in the future. In addition, the number and spacing of dose/concentration levels should also be adequate to fulfil the objectives of the study (e.g. to demonstrate dose response relationships if this is required).

Existing data to be considered

396. Existing data available before consideration of OECD TG 210 might include *in vivo* results obtained with other vertebrates (e.g. a positive *in vivo* assay with amphibians, for example OECD TG 231; or positive findings for thyroid endpoints in mammalian repeat dose toxicity studies, for example OECD TG 407), or one or more of a range of *in silico* or *in vitro* results which suggest that thyroid disruption may occur *in vivo* (but note the limitations of this approach, given that validated *in silico* and *in vitro* screens for thyroid activity are not yet available). Such indicators of possible thyroid activity might include quantitative structure activity relationship (QSAR) predictions of thyroid activity, "read-across" from *in vivo* results obtained with structurally related chemicals or positive results from an *in vitro* screen for thyroid agonist/antagonist activity.

Scenarios: Positive and negative results combined with existing data

397. The scenarios (A to R) presented in Table C.2.8 represent all the possibilities of positive or negative results in combination with the presence or absence of existing data. The action taken will also depend on the regulatory environment, but the considerations given here are generally science based. Wherever possible, the recommended "next step which could be taken" avoids unnecessary animal testing. However, sometimes conducting an animal

test will be indicated and then the relevance of species, strain and exposure route should always be considered. Further considerations specific to each scenario are given in the table.

398. Positive results obtained with one or more of the endpoints (Table C.2.8, Scenarios A-I) will not result in a conclusion that the test chemical is a possible thyroid-acting ED *in vivo*, although this may strengthen such a case. In some cases, this may need to be followed up with more comprehensive testing to show whether adverse apical effects related to thyroid impacts occur in sensitive species such as amphibians at any part of the life cycle (and hence to discover whether the chemical is an ED acting through thyroid mechanisms). In other words, a positive result in OECD TG 210 alone will not trigger further testing. Existing data suggesting endocrine activity will strengthen the case for additional testing.

399. The situation in which OECD TG 210 gives a negative result (Table C.2.8, Scenarios J-R) needs careful consideration of any existing data. If the weight of evidence of these data suggests that the chemical is thyroid-active both *in vitro* and *in vivo* in other species (Scenario J), then the probability is that OECD TG 210 may simply be insufficiently responsive in that case, or fish in general may be unresponsive. In some of these circumstances, it might be appropriate to conduct an Amphibian Metamorphosis Assay (AMA; OECD TG 231) or a Larval Amphibian Growth and Development Assay (LAGDA; OECD TG 241).

400. If OECD TG 210 and existing *in vivo* data are all negative, but *in vitro* data reveal some endocrine activity (Scenario K), the probability is that the test chemical is not sufficiently potent to produce endocrine effects *in vivo* in fish, or it may be rapidly metabolised. In such a situation, further testing will generally not be necessary. If the chemical is known to bioaccumulate slowly, it may be that exposures in the *in vivo* tests were not of sufficient duration, in which case longer term testing might be justified. If existing information, including QSAR predictions, *in vivo* mammalian data and/or *in vitro* data reveal thyroid activity, consideration should be given to conducting the AMA (OECD TG 231) or LAGDA (OECD TG 241), unless exposure of the aquatic environment can be excluded.

401. On the other hand, if OECD TG 210 and the *in vitro* tests are negative, but there are positive existing *in vivo* data (Scenario M), the chemical may not be thyroid-active in fish. In this situation, the relevant existing non-test and *in vitro* or *in vivo* data should be used to guide decisions about whether to conduct any further *in vivo* testing for thyroid activity with the AMA, or the LAGDA if a positive AMA is already available.

402. Finally, a negative OECD TG 210 test, set against a background of negative *in vitro* and *in vivo* data (Scenario N), suggests that the test chemical is not a possible thyroid-acting ED in fish or other vertebrates, and no further testing for thyroid modes of action (MOA) will generally be necessary.

403. In each of the above scenarios, it is possible that existing data will be equivocal, or there may be no existing data (Scenarios C, F-I, L and O-R). This will weaken the conclusions which can be drawn about a negative OECD TG 210 test, and this is reflected in Table C.2.8. However, a lack of mechanistic data on thyroid activity should usually be rectified before any further *in vivo* testing is finally rejected. Indeed, as a general principle, it is desirable to obtain mechanistic data from non-testing and/or *in vitro* testing approaches before any *in vivo* testing which targets endocrine disruption. On the other hand, if OECD TG 210 is positive but existing data are equivocal, this does not necessarily indicate that the test chemical is thyroid-active, and further *in vitro* or *in vivo* testing may be desirable.

There is also the possibility that equivocal mechanistic data may be the result of multiple modes of endocrine action. Under some circumstances, two opposite modes of simultaneous action (e.g. thyroidogenic and anti-thyroidogenic) could, depending on dose, lead to a minimisation or abolition of adverse effects, while in others two different MOA could potentially reinforce effects on certain apical endpoints and/or modify the typical adverse outcome signs related to certain ED MOA. If multiple MOA are suspected, either from the existing results or based on QSAR/read-across/integrated approaches, this situation should be investigated further if needed for regulatory decision making.

404. The scenario in which the results of OECD TG 210 are themselves equivocal has not been dealt with in Table C.2.8, for reasons of brevity. In this context, an equivocal result might be an inconsistent concentration-response (e.g. no effect at a high concentration but effects at a lower concentration), or a result which borders on statistical significance. Without knowing the exact circumstances, reliable advice cannot be given, but the opinions of an experienced ecotoxicologist should be sought. Clearly, however, such equivocal results do not necessarily rule out the existence of *in vivo* thyroid activity. If convincing reasons for false negatives are suspected (e.g. the test chemical is too bulky to be absorbed via the chorion or it requires metabolic activation), the results from OECD TG 210 should not be considered further but other *in vivo* testing may be indicated.

405. In summary, positive results in the OECD TG 210 test may support the case that a chemical is a possible thyroid disrupter, but cannot on their own be used to reach such a conclusion. More thyroid-specific *in vivo* testing would then be necessary to produce a long-term NOEC/ECx and/or to confirm whether or not the chemical is an actual thyroid disrupter with adverse effects *in vivo*. For suspected thyroid-active chemicals, the best available apical test is the LAGDA (OECD TG 241). Negative results in OECD TG 210 do not necessarily mean that the chemical is not a possible thyroid disrupter – a judgement about its endocrine disruption potential and the possible need for additional testing will have to be made based on a weight of evidence evaluation of existing non-test, *in vitro* and *in vivo* data.

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Table C.2.8. **Fish, Early-Life Stage (FELS) Toxicity Test (OECD TG 210): Guidance for scenarios of combinations of results with existing data**

This table represents possible conclusions to be drawn from assay data, and a next step which could be taken if further evidence is required about possible endocrine disrupting properties and/or effects. The guidance offered is not meant to be prescriptive, but provides science-based considerations. It encourages the use of all available data and expert judgement in a weight of evidence approach. Regional and national interpretation of results and "next steps" may vary.

The conclusions are grouped into a series of scenarios (A-R), each scenario representing a different combination of assay results, existing *in vitro* data and existing *in vivo* data. The symbol "+" indicates that the data in question represent a positive result, «-" indicates a negative result, and "Eq/0" indicates that the data are either equivocal or are not available. Note that there are no endpoints in this assay considered to be diagnostic of an estrogen/androgen/thyroid/steroidogenesis (E,A,T,S) modality.

Existing results: * "Mechanism (*in vitro* mechanistic data)" assumes that mechanistic data are available from thyroid hormone receptor (TR) and other assays concerning mechanisms of thyroid disruption, although these are not in common use. In practice, data from all assays may not be available and therefore this must be taken into account when deciding on the "next step". Quantitative structure activity relationship (QSAR) predictions of estrogen and androgen binding/activation may be made for some substances. There is no evidence at present that equivalent *in vitro* assays with systems derived from fish offer advantages over their mammalian counterparts.

Existing results: ** "Effects (*in vivo* effects of concern)" assumes effects have been observed in other *in vivo* screens/tests which give rise to concern that the test chemical may be an endocrine disrupter.

Scenarios	Result of OECD TG 210 assay (FELS)	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
A	+	+	+	Limited evidence for *in vivo* thyroid activity with potential adverse effects (developmental/growth toxicity) in fish, plus thyroid effects in other species	Consider performing an Amphibian Metamorphosis Assay (AMA), or a Larval Amphibian Growth and Development Assay (LAGDA) if a positive AMA result is already available, or if it is regarded as *a priori* likely that an AMA would be positive.	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a thyroid (ant)agonist.
B	+	+	–	Weak evidence for *in vivo* thyroid activity with potential adverse effects (developmental/growth toxicity) in fish or other species.	Consider performing an AMA.	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a thyroid (ant)agonist.
C	+	+	Eq/0	Weak evidence for *in vivo* thyroid activity with potential adverse effects (developmental/growth toxicity) in fish or other species.	Consider performing an AMA.	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a thyroid (ant)agonist. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple modes of action (MOA). If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
D	+	–	+	Limited evidence for endocrine activity which may be thyroid-related, with potential adverse effects (developmental/growth toxicity) in fish, plus thyroid effects in other species.	Consider performing an AMA, or a LAGDA if a positive AMA result is already available.	The lack of *in vitro* thyroid activity is not evidence against any thyroid activity, due to the limited nature of current *in vitro* thyroid screens.
E	+	–	–	No evidence for endocrine activity, but damaged metamorphosis in the Fish, Early-Life Stage (FELS) assay could be caused by thyroid disruption.	Consider performing *in vitro* thyroid assays, quantitative structure activity relationship (QSAR) predictions or read-across if these have not already been conducted.	
F	+	–	Eq/0	No evidence for endocrine activity, but damaged metamorphosis in the FELS assay could be caused by thyroid disruption.	Consider performing *in vitro* thyroid assays if these have not already been conducted. If these are positive, it may be desirable to conduct an AMA.	It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.

Scenarios	Result of OECD TG 210 assay (FELS)	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
G	+	Eq/0	+	Weak evidence for endocrine activity which may be thyroid-related, with potential adverse effects (developmental/growth toxicity) in fish, plus thyroid effects in other species.	Consider performing a new *in vitro* thyroid assay for (ant)agonistic activity, or QSAR predictions or read-across.	If a new *in vitro* mechanistic assay is conducted, note that a negative result does not mean that the test material has no thyroid activity. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
H	+	Eq/0	–	No evidence for endocrine activity, but damaged metamorphosis in the FELS assay could be caused by thyroid disruption.	Consider performing a new *in vitro* thyroid assay for (ant)agonistic activity, or QSAR predictions or read-across.	If a new *in vitro* mechanistic assay is conducted, note that a negative result does not mean that the test material has no thyroid activity. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
I	+	Eq/0	Eq/0	No evidence for endocrine activity, but damaged metamorphosis in the FELS assay could be caused by thyroid disruption.	Consider performing a new *in vitro* thyroid assay for (ant)agonistic activity. If this is positive, it may be desirable to conduct an AMA.	If a new *in vitro* mechanistic assay is conducted, note that a negative result does not mean that the test material has no thyroid activity. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
J	–	+	+	The test chemical may be a thyroid (ant)agonist without activity in fish.	Some regulatory authorities may conclude that no further evidence is required, but it might be desirable to conduct an AMA, or a LAGDA if a positive AMA is already available.	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a thyroid (ant)agonist.
K	–	+	–	The test chemical may a thyroid (ant)agonist without activity *in vivo*.	If there is no activity in fish, amphibians or mammals, further evidence is probably not needed.	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a thyroid (ant)agonist.

Scenarios	Result of OECD TG 210 assay (FELS)	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
L	–	+	Eq/0	The test chemical may a thyroid (ant)agonist without activity *in vivo*.	If there is no activity in fish or mammals, further evidence is probably not needed. However, if the equivocal or absent *in vivo* data relate to amphibians, it may be desirable to repeat an AMA.	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a thyroid (ant)agonist. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
M	–	–	+	The test chemical may be without thyroid activity in fish.	Some regulatory authorities may conclude that no further evidence is required, but it might be helpful to perform an AMA, or a LAGDA if the positive *in vivo* data are from an AMA.	The lack of *in vitro* thyroid activity is not evidence against any thyroid activity, due to the limited nature of current *in vitro* thyroid screens.
N	–	–	–	The test chemical may be without thyroid activity in fish or other taxa.	No further action is necessary.	–
O	–	–	Eq/0	The test chemical may be without thyroid activity in fish or other taxa.	Some regulatory authorities may conclude that no further evidence is required, but if mammalian or amphibian data are absent, it might be desirable to conduct a thyroid-responsive rodent screen (e.g. rat pubertal) or an AMA.	The lack of *in vitro* thyroid activity is not evidence against any thyroid activity, due to the limited nature of current *in vitro* thyroid screens. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
P	–	Eq/0	+	The test chemical may be without thyroid activity in fish.	Some regulatory authorities may conclude that no further evidence is required, but it might be considered worthwhile to conduct an(other) *in vitro* thyroid assay, QSAR prediction or read-across.	If a new *in vitro* mechanistic assay is conducted, note that a negative result does not mean that the test material has no thyroid activity. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.

Scenarios	Result of OECD TG 210 assay (FELS)	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
Q	–	Eq/0	–	The test chemical may be without thyroid activity in fish or other taxa.	No further action is necessary.	It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
R	–	Eq/0	Eq/0	The test chemical may be without thyroid activity in fish.	Some regulatory authorities may conclude that no further evidence is required, but it might be considered worthwhile to conduct an(other) *in vitro* thyroid assay, or QSAR predictions or read-across.	If a new *in vitro* mechanistic assay is conducted, note that a negative result does not mean that the test material has no thyroid activity. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.