C.2.2. 21-Day Fish Assay (OECD TG 230)

Status: Assay validated by the OECD.

297. Modality detected/endpoints: estrogens (♂ VTG ↑; ♂ 2º sex characteristics ↓); anti-estrogens (♀ VTG ↓); androgens (♂ 2º sex characteristics in ♀); anti-androgens (♂ 2º sex characteristics ↓); aromatisable androgens (♂ VTG ↑); aromatase inhibitors (♀ VTG ↓). Note that this assay has low statistical power to identify anti-androgenic activity.

Background to the assay

298. This assay is designed as a screen for the types of *in vivo* endocrine disruption activity in fish which are listed above. The endpoints are indicators of hormonal activity and there are no apical measures of adverse effects **that can be attributed to a single estrogen/androgen/thyroid/steroidogenesis (E,A,T,S) modality** (although it is possible that some substances could cause cessation of spawning). A variation of this assay specifically designed for the detection of androgens and anti-androgens, the Androgenised Female Stickleback Screen (AFSS), is described elsewhere in this document. Anti-androgens may also be detected by the Juvenile Medaka Anti-Androgen Screening Assay (JMASA).

When/why the assay may be used

299. Although data from OECD TG 230 could, in principle, be available at any stage in the hazard assessment process, the most likely scenario will be when there are relatively few data available about the possible endocrine disrupting properties of a chemical. The assay is most likely to be used either as part of a battery of *in vitro* and *in vivo* screens, or to follow up on existing data which suggest possible endocrine disruption activity. It is also possible that no existing endocrine-relevant data are available (i.e. OECD TG 230 has been used as a primary screen), but in that case a positive result in the screen should ideally be followed up with relevant *in vitro* screening in an attempt to confirm the suspected mode of action (MOA). Given the high degree of endocrine system conservation across the vertebrates, endocrine activity in this assay may also indicate the possibility of related activity in other organisms such as amphibians, reptiles, birds or mammals. Possible conclusions to be derived from the results of OECD TG 230, and guidance about potential additional studies to strengthen weight of evidence, are summarised in Table C.2.2.

300. In order to provide information relevant for assessing whether or not a chemical may fulfil the WHO/IPCS (2002) definition of an endocrine disrupter (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

301. Given the commonality of endocrine mechanisms in the vertebrates, relevant existing data available before deployment of OECD TG 230 might include *in vivo* results obtained with other vertebrates (e.g. a Uterotrophic Assay with rodents, positive findings for endocrine endpoints in mammalian repeat dose toxicity or reproductive studies), or one or more of a range of *in silico* or *in vitro* results which suggest that the modalities indicated above may occur *in vivo*. Such indicators of possible *in vivo* activity might include quantitative structure activity relationship (QSAR) predictions of endocrine activity, high throughput screening (HTS) data, "read-across" from *in vivo* results obtained with structurally related chemicals, or positive results from an *in vitro* screen for estrogen or androgen receptor-mediated activity, or for effects on steroidogenesis (especially aromatase inhibition). Further strong indication of *in vivo* estrogenic activity may also be available from an EASZY Assay with transgenic zebrafish embryos.

Scenarios: Positive and negative results combined with existing data

302. The scenarios (A to R) presented in Table C.2.2 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.

303. Positive results obtained with one or more of the endpoints (Table C.2.2, Scenarios A-I) result in the conclusion that the test chemical is a potential ED *in vivo*. This would ideally need to be followed up with more comprehensive testing to show whether adverse apical effects related to endocrine impacts occur at any part of the life cycle (and hence to discover whether the chemical is **an ED acting through E,A,T,S pathways**). In other words, a positive result in OECD TG 230 may trigger TG 234 (Fish Sexual Development Test [FSDT]) at Level 4 or fish life cycle testing at Level 5. Existing data suggesting endocrine activity will strengthen the case for additional testing.

304. The situation in which OECD TG 230 gives a negative result (Table C.2.2, Scenarios J-R) needs careful consideration of any existing data. If the weight of evidence of these data suggests that the chemical is endocrine active both *in vitro* and *in vivo* in other species (Scenario J), then the probability is that OECD TG 230 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 FSDT (OECD TG 234), or

alternatively, a fish life cycle test (either MEOGRT – OECD TG 240, or ZEOGRT) to confirm that there is no endocrine activity in fish.

305. If OECD TG 230 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 adult fish, or it may be rapidly metabolised. However, TG 230 does not include some endpoints which are included in TG 229 (fecundity and histopathology), which is able to detect certain endocrine-active substances not detected by TG 230 alone. In such a situation, further testing may or may not be necessary. A lack of effects in adult fish does not preclude the possibility that endocrine-mediated effects may manifest in fish exposed during a more sensitive life stage (e.g. as embryos or larvae). If the chemical is known to bioaccumulate slowly, it may be that exposures in the *in vivo* tests are not of sufficient duration, in which case longer term testing might be justified. If the *in vitro* data reveal anti-androgenic or thyroid activity, consideration should be given to conducting the Androgenised Female Stickleback Screen (AFSS – OECD GD 148) or Juvenile Medaka Anti-Androgen Screening Assay (JMASA) or the Amphibian Metamorphosis Assay (OECD TG 231), respectively.

306. On the other hand, if OECD TG 230 and the *in vitro* tests are negative but there are positive existing *in vivo* data (Scenario M), the chemical is probably not a potential ED with the modalities listed above, but it may act via estrogen- or androgen-related modes of action (MOA) not covered by the *in vitro* screens, or it may be more potent in species or life stages that have not been tested. In this situation, the relevant existing *in vitro* and *in vivo* data should be used to guide decisions about whether to conduct any further testing, either for modalities such as anti-androgenicity or including life stages represented in OECD TG 234 (FSDT) or in the MEOGRT or ZEOGRT.

307. Finally, a negative OECD TG 230 screen, set against a background of negative *in vitro* and *in vivo* data (Scenario N) **that includes relevant *in vivo* data for fish**, suggests that the test chemical is not a potential ED in fish or other vertebrates via estrogenic, anti-estrogenic, androgenic or steroidogenic MOA, and no further testing will generally be necessary for these modalities. It remains possible that it has anti-androgenic or thyroid activity, although negative *in vitro* tests for these modalities would suggest that this scenario is unlikely.

308. 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 230 test, and this is reflected in Table C.2.2. However, a lack of mechanistic data on endocrine 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 before any *in vivo* testing. On the other hand, if OECD TG 230 is positive, further *in vivo* testing is generally indicated, particularly when existing data are equivocal, or if there are no existing data. 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. estrogenic and anti-estrogenic) could, depending on dose, lead to a minimisation or abolition of effects, while in others two different MOA (e.g. estrogenic and anti-androgenic) could potentially reinforce effects. 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 and, if necessary, the weight given to the apparently equivocal mechanistic data should be increased.

309. The scenario in which the results of OECD TG 230 are themselves equivocal has not been dealt with in Table C.2.2, 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* endocrine activity. For example, vitellogenin (VTG) induction in males at a high concentration might be masked by any systemic toxicity, while VTG depression in females might just fail to reach a statistically significant level because VTG levels were relatively low to begin with. If these or other possible reasons for false negatives are suspected with good reason, the screen could be repeated if none of the test concentrations have given reliable data (e.g. conduct it at lower concentrations which avoid systemic toxicity), or a more appropriate version of it (e.g. ensure females have high VTG levels at the start of the test) could be conducted. However, note that a repeat test in the event of systemic toxicity would not be needed providing at least one tested concentration was not subject to such effects.

310. In summary, positive results in the OECD TG 230 screen indicate that a chemical is a possible endocrine disrupter. More predictive *in vivo* testing would then be necessary to produce a long-term no-observed-effect-concentration/x% effect concentration (NOEC/ECx) and/or to confirm whether or not the chemical is an actual endocrine disrupter with adverse effects *in vivo*. Negative results in OECD TG 230 do not necessarily mean that the chemical is not a potential ED – 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 *in vitro* and *in vivo* data.

Reference

WHO/IPCS (2002), "Global assessment of the state-of-the-science of endocrine disrupters", Damstra, T. et al. (eds.) WHO/PCS/EDC/02.2, World Health Organization, Geneva, www.who.int/ipcs/publications/new_issues/endocrine_disruptors/en.

Table C.2.2. **21-Day Fish Assay (OECD TG 230):**
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 apical endpoints in this assay considered to be diagnostic of an E,A,T,S modality.

Existing results: * "Mechanism (*in vitro* mechanistic data)" assumes that mechanistic data are available from estrogen receptor (ER-), androgen receptor (AR-) and steroidogenesis-based assays (Level 2). Thyroid hormone receptor (TR) and other assays concerning mechanisms of thyroid disruption may be available, but they 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 etrogen 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 230 assay	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	+	+	+	Strong evidence for *in vivo* endocrine activity in fish and other organisms.	Consider performing a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT), especially if the intention is to obtain precise data on a reproductive or developmental no-observed-effect-concentration/x% effect concentration (NOEC/ECx).	An alternative approach would be to deploy TG 234 (Fish Sexual Development Test [FSDT]), especially if sexual development is expected to give a response at lower concentrations than reproduction.
B	+	+	–	Strong evidence for *in vivo* endocrine activity in fish, despite lack of *in vivo* effects in existing tests.	Consider performing a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT), especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx.	An alternative approach would be to deploy OECD TG 234 (FSDT), especially if sexual development is expected to give a response at lower concentrations than reproduction. If the negative *in vivo* data are from a fish test (e.g. OECD TG 229), consider possible reasons for the disparity (e.g. differences in species sensitivity) before conducting a life cycle test.
C	+	+	Eq/0**	Strong evidence for *in vivo* endocrine activity in fish, despite equivocal or absent *in vivo* data in other species.	Consider performing a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT), especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx.	If no existing fish data are available, it may be worth performing OECD TG 234 (FSDT) before a possible life cycle test in order to obtain information on whether sexual development is a sensitive part of the life cycle. Such information could influence the design of the life cycle test. 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	+	–	+	Moderate evidence for *in vivo* endocrine activity in fish and other species, but confidence about MOA is reduced by negative mechanistic data.	Consider performing a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT), especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx.	The negative *in vitro* data suggest that the test chemical may be metabolically activated *in vivo,* or it may operate via mechanisms not covered by the *in vitro* screens. An alternative approach to a life cycle test would be to deploy OECD TG 234 (FSDT), especially if sexual development is expected to give a response at lower concentrations than reproduction.
E	+	–	–	Moderate-strong evidence for *in vivo* endocrine activity in fish, but confidence is reduced by negative *in vitro* data and negative *in vivo* activity in other species.	Consider performing a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT), especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx.	The negative *in vitro* data suggest that the test chemical may be metabolically activated *in vivo*, or it may operate via mechanisms not covered by the *in vitro* screens. An alternative approach to a life cycle test would be to deploy OECD TG 234 (FSDT), especially if sexual development is expected to give a response at lower concentrations than reproduction. If the negative *in vivo* data are from a fish test (e.g. OECD TG 229), consider possible reasons for the disparity (e.g. differences in species sensitivity) before conducting a life cycle test.

Scenarios	Result of OECD TG 230 assay	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)**			
F	+	–	Eq/0	Moderate-strong evidence for *in vivo* endocrine activity in fish, but confidence is reduced by negative *in vitro* data and equivocal or absent *in vivo* activity in other species.	Consider performing a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT), especially if the intention is to obtain precise data on a reproductive or developmental NOEC/ECx.	The negative *in vitro* data suggest that the test chemical may be metabolically activated *in vivo*, or it may operate via mechanisms not covered by the *in vitro* 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. If no existing fish data are available, it may be worth performing OECD TG 234 (FSDT) before a possible life cycle test in order to obtain information on whether sexual development is a sensitive part of the life cycle. Such information could influence the design of the life cycle test.
G	+	Eq/0	+	Strong evidence for *in vivo* endocrine activity in fish, but mechanism unconfirmed.	Obtain mechanistic data, then consider performing a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT).	An alternative approach to a life cycle test would be to deploy OECD TG 234 (FSDT), especially if sexual development is expected to give a response at lower concentrations than reproduction. 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	–	Strong-moderate evidence for *in vivo* endocrine activity in fish, but mechanism unconfirmed.	Obtain mechanistic data, then consider performing a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT).	An alternative approach to a life cycle test would be to deploy OECD TG 234 (FSDT), especially if sexual development is expected to give a response at lower concentrations than reproduction. 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. If the negative *in vivo* data are from a fish test (e.g. OECD TG 229), consider possible reasons for the disparity (e.g. differences in species sensitivity) before possibly conducting a life cycle test.
I	+	Eq/0	Eq/0	Moderate evidence for *in vivo* endocrine activity in fish, but mechanism unconfirmed.	Obtain mechanistic data, then consider performing a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT).	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. If no existing fish data are available, it may be worth performing OECD TG 234 (FSDT) before a possible life cycle test (MEOGRT – OECD TG 240, or ZEOGRT) in order to obtain information on whether sexual development is a sensitive part of the life cycle. Such information could influence the design of the life cycle test.
J	–	+	+	Based on the existing data, the chemical has endocrine activity *in vivo*. The lack of response in OECD TG 230 suggests that fish are not responsive, unless the existing data are from fish.	Consider performing OECD TG 234 (FSDT).	It is possible that the failure to give a positive result in OECD TG 230 was caused by the relatively short exposure time (three weeks). If this is suspected (e.g. the chemical only bioaccumulates slowly), or if the existing *in vivo* data are from a fish, OECD TG 234 (FSDT) or potentially a life cycle test (MEOGRT – OECD TG 240, or ZEOGRT) would be able to study the effects of longer exposure and confirm whether there is a hazard to fish. Choice of test should be guided by the existing *in vivo* data.

Scenarios	Result of OECD TG 230 assay	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)**			
K	–	+	–	There is no evidence that the chemical is a potential ED *in vivo*, probably because it is very weakly acting or rapidly metabolised.	Probably no further action, but see comments in right-hand column.	It is possible that EDs which bioaccumulate slowly may only cause effects *in vivo* after exposure times longer than three weeks. If this is suspected, and depending on which part of the life cycle is suspected of being the most sensitive, consider performing OECD TG 234 (FSDT), or a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT). It is also possible that the chemical may be an anti-androgen *in vivo* (consider performing the Androgenised Female Stickleback Screen [AFSS] or Juvenile Medaka Anti-Androgen Screening Assay [JMASA]), or a thyroid-active chemical *in vivo* (consider performing the Amphibian Metamorphosis Assay [AMA] – OECD TG 231, or *Xenopus* Embryo Thyroid Signalling Assay [XETA]).
L	–	+	Eq/0	The chemical may not be an ED *in vivo*, but the confidence in this conclusion is relatively low as there is only one unequivocal *in vivo* test result (a negative).	Probably no further action, but see comments in right-hand column.	If the equivocal existing data are from a fish assay, consider performing a fish assay (OECD TG 229 or TG 230) with a different species, or a longer term test (TG 234 [FSDT] or life cycle [MEOGRT – OECD TG 240, or ZEOGRT]) if the chemical is a slow bioaccumulator. It is also possible that the chemical may be an anti-androgen *in vivo* (consider performing the AFSS or JMASA), or a thyroid-active chemical *in vivo* (consider performing the AMA – OECD TG 231, or XETA). 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 chemical is apparently not a potential ED in fish but it does have activity in other species.	Use the existing *in vivo* data to help decide whether a longer term test with an appropriate fish species is indicated.	It is also possible that the chemical may be an anti-androgen *in vivo* (consider performing the AFSS or JMASA), or a thyroid-active chemical *in vivo* (consider performing the AMA – OECD TG 231, or XETA), although lack of *in vitro* binding affinity with the androgen receptor suggests the former is unlikely. Use the existing *in vivo* data to guide any further testing.
N	–	–	–	The chemical is probably not a potential ED *in vivo*.	No further action with respect to estrogenic, anti-estrogenic, androgenic or steroidogenic MOA.	It is still possible that the chemical may be an anti-androgen *in vivo* (consider performing the AFSS or JMASA), or a thyroid-active chemical *in vivo* (consider performing the AMA – OECD TG 231, or XETA), although lack of *in vitro* binding affinity with the androgen receptor suggests the former is unlikely.

Scenarios	Result of OECD TG 230 assay	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)**			
O	–	–	Eq/0	The chemical is probably not a potential ED in fish.	Probably no further action. However, see comments in right-hand column.	If the paucity of *in vivo* data is a concern, performance of a screening test (OECD TG 229 or TG 230) with a different species, or a longer term test (i.e. TG 234 [FSDT] or life cycle) could be considered. It is still possible that the chemical may be an anti-androgen *in vivo* (consider performing the AFSS or JMASA), or a thyroid-active chemical *in vivo* (consider performing the AMA – OECD TG 231, or XETA), although lack of *in vitro* binding affinity with the androgen receptor suggests the former is unlikely. 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 chemical is probably not a potential ED in fish, but confidence in this conclusion is low given the lack of mechanistic *in vitro* data and the availability of positive existing *in vivo* data.	Obtain mechanistic data, then consider whether further testing is desirable.	If the mechanistic data confirm that the chemical has potential endocrine action, consider conducting a fish assay (OECD TG 229 or TG 230) with another species, or a longer term test (OECD TG 234 [FSDT] or life cycle). Use the existing *in vivo* data as a guide to test choice. If the mechanistic data reveal anti-androgenic or thyroid activity, consider performing the AFSS or JMASA), or a thyroid-active chemical *in vivo* (consider performing the AMA – OECD TG 231, or XETA), respectively. 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.
Q	–	Eq/0	–	The chemical is probably not a potential ED in fish, but the lack of mechanistic *in vitro* data are a concern, even though the existing *in vivo* data are negative.	Obtain mechanistic data, then consider whether further testing is desirable.	If the mechanistic data confirm that the chemical has potential endocrine action, consider conducting a fish assay (OECD TG 229 or TTG 230) with another species, or a longer term test (OECD TG 234 [FSDT]) or life cycle). Use the existing *in vivo* data as a guide to test choice. If the mechanistic data reveal anti-androgenic or thyroid activity, consider performing the AFSS or JMASA) or a thyroid-active chemical *in vivo* (consider performing the AMA – OECD TG 231, or XETA), respectively. 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 230 assay	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)**			
R	–	Eq/0	Eq/0	The chemical is probably not a potential ED in fish, but confidence in this conclusion is low given the lack of mechanistic *in vitro* and existing *in vivo* data.	Obtain mechanistic data, then consider whether further testing is desirable.	If the mechanistic data confirm that the chemical has potential endocrine action, consider conducting a fish assay (OECD TG 229 or TG 230) with another species, or a longer term test (OECD TG 234 [FSDT] or life cycle). Use the existing *in vivo* data as a guide to test choice. If the mechanistic data reveal anti-androgenic or thyroid activity, consider performing the AFSS or JMASA), or a thyroid-active chemical *in vivo* (consider performing the AMA – OECD TG 231, or XETA), respectively. 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.