

C.2.1. Fish Short-Term Reproduction Assay (FSTRA) (OECD TG 229)

Status: Assay validated by the OECD.

282. Modality detected/endpoints: estrogens (♂ VTG ↑; ♂ 2° sex characteristics ↓); anti-estrogens (♀ VTG ↓); androgens (♂ 2° sex characteristics in ♀); anti-androgens (♂ 2° sex characteristics ↓); aromatisable androgens (♂ VTG ↑); aromatase inhibitors (♀ VTG ↓); non-specific effects on hypothalamic/pituitary/gonadal (HPG) axis, plus other reprotox (fecundity ↓); (optional endpoint – gonadal histo-pathology. This may assist with diagnosis of mode of action). Note that this assay may, in some cases, have low statistical power or sensitivity to detect anti-androgenic activity through effects on secondary sexual characteristics. However, if gonad histopathology has been optionally studied, changes in Leydig cells resulting from anti-androgen exposure may have been observed. Finally, diagnostic endpoints (i.e. indicators of hormonal activity) and the apical endpoint (i.e. fecundity) should be considered together to obtain maximum value from this assay.

Background to the assay

283. This assay is primarily designed as a screen for the types of *in vivo* endocrine disruption activity in fish which are listed above, but it also provides information on adverse effects on fecundity which could be used in characterising the hazards of an individual chemical based on a predicted environmental concentration/predicted no-effect concentration approach (although note that only three test concentrations are normally used, so precision of a no-observed-effect-concentration/x% effect concentration (NOEC/ECx) may be relatively low). The fecundity endpoint, which although not necessarily diagnostic of endocrine action, does indicate that apical effects on reproduction are occurring, is sensitive to known endocrine disruptors (EDs). However, the validation studies demonstrated high variability for fecundity (and consequently low power to detect an effect) under certain suboptimal test conditions (e.g. for some fish strains, the recommended degree of replication may provide low power). If the assay gives a positive result, this may be due to a positive indicator of hormonal activity (vitellogenin level, secondary sexual characteristic development), which may or may not be associated with decrease in fecundity. Each of these three possible combinations of positive response should be considered separately (although the distinctions between indicators of hormonal activity and apical effects are not always clear), so they have been listed individually as points 1, 2 and 3 in the possible conclusions column of [Table C.2.1](#). Although this test guideline (TG) is primarily a screening assay where a combination of positive data on hormonal activity and fecundity could lead to a conclusion that higher level testing is desirable (depending on the overall weight of evidence), some regulatory authorities may consider that such a combination is sufficient evidence on its own of endocrine disruption providing an effect on fecundity that is sufficiently large enough to constitute a plausible threat to a fish population. It should be noted, in addition, that due to the relatively short exposure time employed in this screen (three weeks), effects of some chemicals on fecundity might not be as apparent as in longer term exposures, especially for bioaccumulative chemicals. Also, as only three test concentrations are employed, even a reliable short-term NOEC or ECx for fecundity cannot be precisely derived.

When/why the assay may be used

284. Although OECD TG 229 could, in principle, be used at any stage in the hazard assessment process, the most likely use scenario will be when there are relatively few data available about the possible endocrine disrupting properties of a chemical. The results from this assay are most likely to be available after deployment of a battery of *in vitro* and *in vivo* screens (e.g. the United States Environmental Protection Agency's Endocrine Disrupter Screening Program), or as a supplement to existing data which suggest possible endocrine disruption activity. It is also possible that no existing endocrine-relevant data are available (i.e. OECD TG 229 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 to investigate the suspected mode of action (MOA). Given the high degree of endocrine system conservation across the vertebrates, adverse endocrine-linked effects in the FSTRA may also indicate the possibility of related activity in other organisms such as amphibians, reptiles, birds or mammals.

285. 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 (GD) is not the place to address this issue directly, but it should be considered when EAS-sensitive 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

286. Given the commonality of endocrine mechanisms in the vertebrates, relevant existing data available before deployment of OECD TG 229 might include *in vivo* results obtained with other vertebrates (e.g. a positive 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. OECD TG 229 may itself also be used as part of a battery of screening assays. Conduct of OECD TG 229 would be particularly relevant if knowledge is sought about the test chemical's effects on the mature reproductive phase of the fish life cycle (as opposed to effects on the immature sexual development phase), because it provides some apical information on reproductive success and gonad

histopathology. However, this assay is also likely to be responsive to many chemicals which act primarily on sexual development.

Scenarios: Positive and negative results combined with existing data

287. The scenarios (A to R) presented in [Table C.2.1](#) 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.

288. Positive results obtained with one or more of the indicators of hormonal activity (Scenarios A-I, sub-section 2) result in the conclusion that the test chemical is a potential ED *in vivo*. If both an indicator of hormonal activity and fecundity give a response (Scenarios A-I, sub-section 1), this provides strong evidence for *in vivo* endocrine activity on the HPG axis with potential adverse effects, and some regulatory authorities may consider that this is sufficient evidence of ED. If only fecundity responds (Scenarios A-I, sub-section 3), it suggests that the chemical is a reproductive or general systemic toxicant, with a reduced probability that it is an ED **that acts on one or more of the endocrine modalities covered in the Conceptual Framework** (although existing positive *in vitro* data, or positive *in vivo* data from other species, would have to be weighed against this conclusion).

289. As indicated above, although a combined effect on fecundity and an indicator of hormonal activity in OECD TG 229 suggests that the test chemical is a reproductive toxicant acting through one or more estrogen/androgen/thyroid/steroidogenesis (E,A,T,S) pathways (assuming that the concentration giving this response is not sufficiently high to cause systemic toxicity), a result of this type may need to be followed up with a more comprehensive reproduction test if countries need further evidence (e.g. a Medaka Extended One-Generation Reproduction Test [MEOGRT], OECD TG 240; or Zebrafish Extended One-Generation Reproduction Test [ZEOGRT]) which is able to provide a more reliable and reproducible NOEC or EC_x for adverse effects. An exception might be if there are no indications of endocrine activity (either from this or other screens/tests), although in such a case, an NOEC or EC_x for reproductive effects would still need to be derived for a non-endocrine hazard identification/characterisation (e.g. using data from OECD TG 210). Equally, if one or more biomarkers for hormonal activity alone respond **without a corresponding response from apical endpoints**, this would also need to be followed up with more comprehensive testing to show whether any adverse apical effects occur at other parts of the life cycle, if countries need further evidence whether the chemical is an ED. In other words, in order to strengthen weight of evidence (WOE) in relation to ED, a positive result of whichever type in OECD TG 229 could be followed by fish partial or full life cycle testing at Level 4 or 5. Existing data suggesting endocrine-specific activity (e.g. positive *in vitro* data, or positive *in vivo* data from other species) will strengthen the case for additional testing still further if the intention is to establish a firmer link between endocrine activity and adverse effects.

290. The situation in which OECD TG 229 gives a negative result (Table C.3.1, Scenarios J-R) needs careful consideration of the WOE based on any existing data. If these data suggest that the chemical is endocrine active both *in vitro* and *in vivo* (Scenario J), then it is possible that OECD TG 229 is simply insufficiently sensitive, perhaps due to rapid metabolism, or because the main mode of action (MOA) acts more potently during sexual development, or because fish in general are simply insensitive to the chemical under

consideration. In some of these circumstances, it might therefore be appropriate to conduct further studies, e.g. of metabolism and characterisation of metabolites for endocrine disruptive properties of the chemical in the tested fish species, or a Fish Sexual Development Test (FSDT) (OECD TG 234), or alternatively, a MEOGRT or ZEOGRT to confirm that there is no endocrine activity in fish.

291. If OECD TG 229 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 (e.g. because it is rapidly hydrolysed or metabolised to ED-inactive metabolites). In such a situation, further testing in fish is probably not necessary. However, if the chemical is known to bioaccumulate slowly, it may be that exposures in the *in vivo* tests have been insufficiently prolonged, in which case longer term testing might be justified. Equally, if the *in vitro* or histopathology data reveal anti-androgenic or thyroid activity, consideration may be given to conducting the Androgenised Female Stickleback Screen or Juvenile Medaka Anti-androgen Screening Assay (JMASA), or the Amphibian Metamorphosis Assay (OECD TG 231) or *Xenopus* Embryonic Thyroid Signalling Assay (XETA), respectively.

292. On the other hand, if OECD TG 229 and the *in vitro* tests are negative (Scenario M), but there are positive existing *in vivo* data, the nature of those existing data should be considered. Unless the existing data are from fish, there is no evidence that the chemical is an ED acting on fish reproduction, but it may act via MOA not covered by the *in vitro* screens, or it may be more potent in species or life stages that have not been tested. Finally, if it does cause endocrine activity-related effects in the test but no effects on fecundity, this may simply be due to the lack of sensitivity of this screening test which, as mentioned above, has limitations due to relatively high variability of the fecundity parameter in combination with the relatively low number of fish per exposure concentration, etc. In this situation, the existing *in vivo* data should be used to guide decisions about whether to conduct any further testing, either for modalities such as anti-androgenicity or thyroid activity, or including life stages represented in OECD TG 234 (FSDT) or in the MEOGRT (OECD TG 240) or ZEOGRT.

293. Finally, a negative OECD TG 229 screen, set against a background of negative *in vitro* and *in vivo* data (Scenario N), suggests that the test chemical is not an ED acting on reproduction in fish, and no further testing for estrogenic, anti-estrogenic, androgenic or steroidogenic MOA will generally be necessary. It remains possible that it has anti-androgenic or thyroid activity, although **this scenario is unlikely if relevant *in vitro* tests for these modalities have shown negative results and if no effects have been detected** by gonadal histopathology. However, it should be noted that a full suite of *in vitro* thyroid assays is not yet available.

294. 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 229 test, and this is reflected in [Table C.2.1](#). However, a lack of mechanistic data on endocrine activity should ideally be rectified before any further *in vivo* testing is finally rejected. Indeed, as a general principle, it is desirable to obtain as many relevant ED-related mechanistic non-test and *in vitro* data as possible before doing any *in vivo* testing. On the other hand, if OECD TG 229 is positive, further *in vivo* testing may be needed to establish a more precise NOEC or EC_x for any adverse effects, even if all other existing data are equivocal, or if there are no existing data. Again, however, it will always be desirable to obtain some mechanistic information before conducting further *in vivo* testing. 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 adverse effects, while in others two different MOA (e.g. estrogenic and anti-androgenic) could potentially reinforce effects on certain apical endpoints. 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.

295. The scenario in which the results of OECD TG 229 are themselves equivocal has not been dealt with in [Table C.2.1](#), 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 induction in males at a high concentration might be masked by any systemic toxicity, while fecundity depression might just fail to reach a statistically significant level because the sometimes high variability of this endpoint combined with a relatively small sample size might have reduced the power of the test to detect a difference from the controls. If these or other possible reasons for false negatives are suspected with good reason, the screen could be repeated (e.g. conduct it at lower concentrations which avoid systemic toxicity) or a more appropriate version of it (e.g. more fish per replicate) could be designed and 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 toxicity.

296. In summary, positive results in the OECD TG 229 screen indicate that a chemical is either a reproductive toxicant, or a possible endocrine disrupter, or both. In most cases, more comprehensive *in vivo* testing would be needed if the intention is to derive a long-term NOEC/ECx and/or to confirm whether or not the chemical is an actual endocrine disrupter. In this connection, it should also be borne in mind that effects solely on fecundity might be caused by systemic toxicity rather than endocrine disruption or specific reproductive toxicity, if test concentrations were very high. Negative results in OECD TG 229 do not necessarily mean that the chemical is not a potential ED – a judgement about the endocrine disruption potential and the possible need for additional testing will have to be made in the light 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.2.1. **Fish Short-Term Reproduction Assay (FSTRA) (OECD TG 229):
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.

The assay under discussion could either be positive for both apical endpoints and indicators of endocrine activity, or positive just for apical endpoints, or positive just for indicators of endocrine activity. For each scenario, each of these three possibilities is addressed separately in the possible conclusions column, **taking into consideration other existing data**.

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 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 229 assay	Existing results		Possible conclusions: 1) Indicators of endocrine activity and apical endpoints positive 2) Indicators of endocrine activity positive and apical endpoints negative 3) Indicators of endocrine activity negative and apical endpoints positive	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (<i>in vitro</i> mechanistic data)*	Effects (<i>in vivo</i> effects of concern)**			
A	+	+	+	1) Strong evidence for <i>in vivo</i> endocrine activity with potential adverse effects (reproductive toxicity) in fish. 2) Strong evidence for <i>in vivo</i> endocrine activity in fish. 3) Evidence for <i>in vivo</i> endocrine activity in other species, and strong evidence for reproductive toxicity in fish.	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 the Fish Sexual Development Test (FSDT – OECD TG 234), especially if sexual development is expected to give a response at lower concentrations than reproduction.
B	+	+	–	1) Strong-moderate evidence for <i>in vivo</i> endocrine activity with potential adverse effects (reproductive toxicity) in fish. 2) Strong-moderate evidence for <i>in vivo</i> endocrine activity in fish. 3) Moderate-weak evidence for <i>in vivo</i> endocrine activity, and strong evidence for reproductive toxicity in fish.	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 the FSDT, especially if sexual development is expected to give a response at lower concentrations than reproduction. If the negative <i>in vivo</i> data are from another fish endocrine assay, consider possible reasons for the disparity (e.g. differences in species sensitivity) before conducting a life cycle test.
C	+	+	Eq/0	1) Moderate evidence for <i>in vivo</i> endocrine activity with potential adverse effects in fish. 2) Moderate evidence for <i>in vivo</i> endocrine activity in fish. 3) Weak evidence for <i>in vivo</i> endocrine activity, and strong evidence for reproductive toxicity in fish.	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 the FSDT, especially if sexual development is expected to give a response at lower concentrations than reproduction. If no existing fish data are available, it may be worth performing an FSDT before a life cycle test in order to obtain information on whether sexual development is the most 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	+	–	+	1) Moderate evidence for <i>in vivo</i> endocrine activity with potential adverse effects in fish. 2) Moderate evidence for <i>in vivo</i> endocrine activity in fish. 3) Evidence for <i>in vivo</i> endocrine activity in other species, and strong evidence for reproductive toxicity in fish.	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 <i>in vitro</i> data suggest that the test chemical may be metabolically activated <i>in vivo</i> or may not act via the screened receptor. An alternative approach would be to deploy the FSDT, especially if sexual development is expected to give a response at lower concentrations than reproduction.

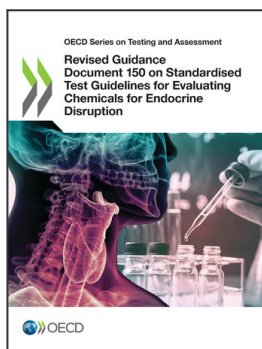
Scenarios	Result of OECD TG 229 assay	Existing results		Possible conclusions: 1) Indicators of endocrine activity and apical endpoints positive 2) Indicators of endocrine activity positive and apical endpoints negative 3) Indicators of endocrine activity negative and apical endpoints positive	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (<i>in vitro</i> mechanistic data)*	Effects (<i>in vivo</i> effects of concern)**			
E	+	–	–	1) Moderate-strong evidence for <i>in vivo</i> endocrine activity with potential adverse effects in fish. 2) Moderate-strong evidence for <i>in vivo</i> endocrine activity in fish. 3) Weak-moderate evidence for <i>in vivo</i> endocrine activity, and strong evidence for reproductive toxicity in fish.	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 <i>in vitro</i> data suggest that the test chemical may be metabolically activated <i>in vivo</i> . An alternative approach would be to deploy the FSDT, especially if sexual development is expected to give a response at lower concentrations than reproduction. If the negative <i>in vivo</i> data are from another fish endocrine assay, consider possible reasons for the disparity (e.g. differences in species sensitivity) before conducting a life cycle test.
F	+	–	Eq/0	1) Moderate-strong evidence for <i>in vivo</i> endocrine activity with potential adverse effects in fish. 2) Moderate-strong evidence for <i>in vivo</i> endocrine activity in fish. 3) Weak evidence for <i>in vivo</i> endocrine activity, and strong evidence for reproductive toxicity in fish.	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 <i>in vitro</i> data suggest that the test chemical may be metabolically activated <i>in vivo</i> . If no existing fish data are available, it may be worth performing an FSDT before a life cycle test in order to obtain information on whether sexual development is the most 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 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.
G	+	Eq/0	+	1) Strong evidence for <i>in vivo</i> endocrine activity with potential adverse effects in fish, but mechanism unconfirmed. 2) Strong evidence for <i>in vivo</i> endocrine activity in fish, but mechanism unconfirmed. 3) Moderate evidence for <i>in vivo</i> endocrine activity (mechanism unconfirmed), and strong evidence for reproductive toxicity in fish.	Obtain more predictive mechanistic data and then consider performing a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT).	An alternative approach would be to deploy the 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.

Scenarios	Result of OECD TG 229 assay	Existing results		Possible conclusions: 1) Indicators of endocrine activity and apical endpoints positive 2) Indicators of endocrine activity positive and apical endpoints negative 3) Indicators of endocrine activity negative and apical endpoints positive	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (<i>in vitro</i> mechanistic data)*	Effects (<i>in vivo</i> effects of concern)**			
H	+	Eq/0	–	1) Moderate-strong evidence for <i>in vivo</i> endocrine activity with potential adverse effects in fish, but mechanism unconfirmed. 2) Moderate-strong evidence for <i>in vivo</i> endocrine activity in fish, but mechanism unconfirmed. 3) Weak-moderate evidence for <i>in vivo</i> endocrine activity (mechanism unconfirmed), and strong evidence for reproductive toxicity in fish.	Obtain more predictive mechanistic data and then consider performing a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT).	An alternative approach would be to deploy the FSDT, especially if sexual development is expected to give a response at lower concentrations than reproduction. If the negative <i>in vivo</i> data are from a fish test (e.g. the 21-day fish assay), consider possible reasons for the disparity (e.g. differences in species sensitivity) before conducting a 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 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	1) Moderate evidence for <i>in vivo</i> endocrine activity with potential adverse effects in fish, but mechanism unconfirmed. 2) Moderate evidence for <i>in vivo</i> endocrine activity in fish, but mechanism unconfirmed. 3) Weak evidence for <i>in vivo</i> endocrine activity (mechanism unconfirmed), but strong evidence for reproductive toxicity in fish.	Obtain more predictive mechanistic data and then consider performing a fish life cycle test (MEOGRT – OECD TG 240, or ZEOGRT).	If no existing fish data are available, it may be worth performing a FSDT before a life cycle test in order to obtain information on whether sexual development is the most 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 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	–	+	+	Based on the existing data, the chemical has endocrine activity <i>in vivo</i> . The lack of response in OECD TG 229 suggests that sexually mature fish are not responsive, unless the existing data are from fish.	If existing <i>in vivo</i> data are from fish, consider performing an FSDT (OECD TG 234) (unless reproduction is known to be the most sensitive life stage).	

Scenarios	Result of OECD TG 229 assay	Existing results		Possible conclusions: 1) Indicators of endocrine activity and apical endpoints positive 2) Indicators of endocrine activity positive and apical endpoints negative 3) Indicators of endocrine activity negative and apical endpoints positive	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (<i>in vitro</i> mechanistic data)*	Effects (<i>in vivo</i> effects of concern)**			
K	–	+	–	There is no evidence that the chemical is an endocrine disrupter (ED) <i>in vivo</i> , probably because it is very weakly acting, rapidly metabolised or simply does not reach the target site.	Probably no further action, but see comments in right-hand column.	It is possible that EDs which bioaccumulate slowly may only cause effects <i>in vivo</i> 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 an FSDT, a MEOGRT or a ZEOGRT. It is also possible that the chemical may be an anti-androgen <i>in vivo</i> (consider performing the Androgenised Female Stickleback Screen, or Juvenile Medaka Anti-Androgen Screening Assay [JMASA]), or a thyroid-active chemical <i>in vivo</i> (consider performing the Amphibian Metamorphosis Assay [AMA] OECD TG 231, or <i>Xenopus</i> Embryo Thyroid Signalling Assay [XETA]).
L	–	+	Eq/0	The chemical may not be an ED <i>in vivo</i> , but the confidence in this conclusion is relatively low as there is only one unequivocal <i>in vivo</i> test result (a negative).	If the existing <i>in vivo</i> data are equivocal and from a fish, consider performing a fish assay (OECD TG 229 or TG 230) with a different species, or consider a longer term test (TG 234 [FSDT] or life cycle (EOGRT or ZEOGRT)).	It is also possible that the chemical may be an anti-androgen <i>in vivo</i> (consider performing the Androgenised Female Stickleback Screen, or JMASA), or a thyroid-active chemical <i>in vivo</i> (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 probably not an ED acting on reproduction in fish. However, it has endocrine activity in another species and may act through MOA not covered by the available <i>in vitro</i> assays, or it may be more potent in a species other than that tested, or over a longer exposure period.	If further evidence is required, consider using the existing <i>in vivo</i> data to help choose a longer term test with an appropriate species.	It is also possible that the chemical may be an anti-androgen <i>in vivo</i> (consider performing the Androgenised Female Stickleback Screen, or JMASA), or a thyroid-active chemical <i>in vivo</i> (consider performing the AMA – OECD TG 231, or XETA), although lack of <i>in vitro</i> binding affinity with receptors suggests this is unlikely. Use the existing <i>in vivo</i> data to guide any further testing.
N	–	–	–	The chemical is probably not an ED acting on reproduction in fish. There is a possibility that the chemical is able to affect sexual development in fish, but the probability of this is low given the apparent absence of estrogenic, androgenic or steroidogenic properties.	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 <i>in vivo</i> (consider performing the Androgenised Female Stickleback Screen, or JMASA), or a thyroid-active chemical <i>in vivo</i> (consider performing the AMA – OECD TG 231, or XETA), although lack of <i>in vitro</i> binding affinity with receptors suggests this is unlikely.

Scenarios	Result of OECD TG 229 assay	Existing results		Possible conclusions: 1) Indicators of endocrine activity and apical endpoints positive 2) Indicators of endocrine activity positive and apical endpoints negative 3) Indicators of endocrine activity negative and apical endpoints positive	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (<i>in vitro</i> mechanistic data)*	Effects (<i>in vivo</i> effects of concern)**			
O	–	–	Eq/0	The chemical is probably not an ED acting on reproduction in fish. There is a possibility that the chemical is able to affect sexual development in fish, but the probability of this is low given the apparent absence of estrogenic, androgenic or steroidogenic properties.	Probably no further action. However, see comments in right-hand column.	If the paucity of <i>in vivo</i> data are 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 – MEOGRT or ZEOGRT) could be considered. It is also possible that the chemical may be an anti-androgen <i>in vivo</i> (consider performing the Androgenised Female Stickleback Screen, or JMASA), or a thyroid-active chemical <i>in vivo</i> (consider performing the AMA – OECD TG 231, or XETA), although lack of <i>in vitro</i> binding affinity with receptors suggests this 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 may not be an ED acting on reproduction in fish, but confidence in this conclusion is low given the lack of mechanistic <i>in vitro</i> data and the availability of positive existing <i>in vivo</i> data.	Obtain more predictive mechanistic data, then consider further testing.	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 (TG 234 [FSDT] or life cycle – MEOGRT or ZEOGRT). Use the existing <i>in vivo</i> data as a guide to test design. If the mechanistic data reveal anti-androgenic or thyroid activity, perform the Androgenised Female Stickleback Screen or JMASA, or 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 229 assay	Existing results		Possible conclusions: 1) Indicators of endocrine activity and apical endpoints positive 2) Indicators of endocrine activity positive and apical endpoints negative 3) Indicators of endocrine activity negative and apical endpoints positive	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (<i>in vitro</i> mechanistic data)*	Effects (<i>in vivo</i> effects of concern)**			
Q	–	Eq/0	–	The chemical is probably not an ED acting on reproduction in fish, but the lack of more predictive mechanistic data are a concern, even though the existing <i>in vivo</i> data are negative.	Obtain more predictive mechanistic data, then consider further testing.	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 (TG 234 [FSDT] or life cycle – MEOGRT or ZEOGRT). Use the existing <i>in vivo</i> data as a guide to test design. If the mechanistic data reveal anti-androgenic or thyroid activity, perform the Androgenised Female Stickleback Screen or JMASA, or 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.
R	–	Eq/0	Eq/0	The chemical is probably not an ED acting on reproduction in fish, but confidence in this conclusion is low given the lack of mechanistic <i>in vitro</i> and existing <i>in vivo</i> data.	Obtain more predictive mechanistic data, then consider further testing.	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 (TG 234 [FSDT] or life cycle – MEOGRT or ZEOGRT). Use the existing <i>in vivo</i> data as a guide to test design. If the mechanistic data reveal anti-androgenic or thyroid activity, perform the Androgenised Female Stickleback Screen or JMASA, or 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.



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