C.2.4. *Potamopyrgus antipodarum* Reproduction Test (OECD TG 242)

Status: Assay validated by the OECD.

328. Modality detected/endpoints: This medium-term reproduction *in vivo* assay with the parthenogenetic female mudsnail *Potamopyrgus antipodarum*, a prosobranch mollusc, is expected to be responsive *inter alia* to retinoid X receptor (RXR) (ant)agonists which can interfere with reproduction in molluscs. It may also respond to certain vertebrate steroid agonists (e.g. estradiol) (Duft et al., 2007), but is not recommended for use in this regard as it was not validated with such chemicals except for the androgen agonist trenbolone (to which it did not respond). It exposes the test organisms for less than a whole generation. It is important to note, however, that none of the endpoints in this apical test are specifically responsive to endocrine-active chemicals, and the assay will give positive results with many other substances. The lack of mechanistic assays for endocrine activity in molluscs will prevent firm conclusions about whether test chemicals are endocrine disrupters (EDs) in this taxon, but the data from the test may nevertheless be of value for classification and hazard identification/characterisation.

Background to the assay

329. This assay is run with the parthenogenetic mudsnail *Potamopyrgus antipodarum*. Adult females are exposed to a range of dilutions of the test chemical for 28 days, after which the numbers of ovoviviparous embryos in the brood pouch are measured. Mortality of the adults is also recorded but does not constitute an endpoint likely to be sensitive to endocrine-active chemicals.

When/why the assay may be used

330. Although OECD TG 242 could, in principle, be used at any stage in the hazard assessment process, the most likely use scenario will be when there are already some *in vitro* data available about the possible endocrine activity of a chemical. However, many chemicals with non-endocrine action will also give positive responses in OECD TG 242.

331. The precise modes of action (MOA) of endocrine-active chemicals in molluscs are unknown, and even the MOA of the well-known mollusc ED tributyltin (TBT) is not fully understood, although it appears to act at least partly via the RXR receptor. OECD TG 242 should therefore not be deployed as a primary screen for endocrine activity, because of its lack of specificity. Furthermore, it should be noted that there are no standardised *in vitro* screens for RXR agonists, although some are described in the scientific literature (e.g. Li, Ma and Wang, 2008). As *P. antipodarum* reproduces parthenogenetically, endocrine-active chemicals may produce different responses in sexually reproducing species.

332. 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

333. Existing information on endocrine-related effects from other molluscs should also be considered before deployment of OECD TG 242, but no short-term screens with this phylum have been internationally standardised. Existing data available might also include one or more of a range of *in silico* or *in vitro* results which suggest that endocrine disruption may occur *in vivo* (but note the limitations of this approach, as indicated above). Such indicators of possible activity might include quantitative structure activity relationship (QSAR) predictions, "read-across" from *in vivo* results obtained with structurally related chemicals or positive results from an *in vitro* screen.

Scenarios: Positive and negative results combined with existing data

334. The scenarios (A to R) presented in Table C.2.4 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.

335. Positive results obtained with OECD TG 242 (Table C.2.4, Scenarios A-I) result in the conclusion that the test chemical has adverse apical effects, at least in parthenogenetic molluscs, but these are not necessarily caused by endocrine activity. However, although a positive response of OECD TG 242 indicates that the chemical has adverse effects in parthenogenetic molluscs, it should be noted that many mollusc species such as *Lymnaea stagnalis* have a sexual reproductive strategy and so may respond differently to *P. antipodarum*. Therefore, if countries need further evidence concerning growth and sexual development, etc. in this phylum, a *Lymnaea stagnalis* Reproduction Test (OECD TG 243) would be able to provide information on adverse effects in such mollusc groups. In other words, in order to strengthen weight of evidence, a positive result in OECD TG 242 could be followed by TG 243 (Level 4). 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.

336. The situation in which OECD TG 242 gives a negative result (Table C.2.4, Scenarios J-R) needs careful consideration of 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 242 is simply insufficiently sensitive.

337. If OECD TG 242 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 disruption *in vivo* in molluscs, or it may be rapidly metabolised. In such a situation, further testing is probably not necessary.

338. On the other hand, if OECD TG 242 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 another mollusc, the chemical is possibly not endocrine active in molluscs, but it may be more potent in sexually reproducing species (e.g. *L. stagnalis*) or life stages that have not been tested. In this situation, the existing *in vivo* data should be used to guide decisions about whether to conduct any further testing (e.g. with OECD TG 243).

339. Finally, a negative OECD TG 242, set against a background of negative *in vitro* and *in vivo* data (Scenario N), suggests that the test chemical is probably not endocrine active *in vitro* or *in vivo*, and further action is unnecessary.

340. In each of the above scenarios, it is possible that existing data will be equivocal, or there may be no existing data. This will weaken the conclusions which can be drawn about a negative OECD TG 242, and this is reflected in Table C.2.4. However, a lack of mechanistic data on endocrine activity should ideally be rectified before any further *in vivo* testing is finally conducted, although as indicated above, *in vitro* endocrine screens for molluscs have not yet been internationally standardised. On the other hand, if OECD TG 242 is positive, further *in vivo* testing would generally be needed to quantify any adverse effects in other molluscs, even if all existing data are equivocal, or if there are no existing data. Again, however, it may be useful to obtain some mechanistic information before conducting further *in vivo* testing. Under some circumstances, two opposite modes of simultaneous action could, depending on dose, lead to a minimisation or abolition of adverse effects, while in others two different MOA could potentially reinforce effects on the OECD TG 242 endpoint. 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.

341. The scenario in which the results of OECD TG 242 are themselves equivocal has not been dealt with in Table C.2.4, 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, although note that such responses to ED are sometimes repeatably observed *in vivo*), 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. If possible reasons for false negatives are suspected, OECD TG 242 could be repeated (e.g. conduct it at lower concentrations which avoid systemic toxicity).

342. In summary, positive results in OECD TG 242 indicate that a chemical has adverse effects in molluscs which may or may not be via endocrine activity. This may need to be followed up with further apical testing with sexually reproducing molluscs such as *L. stagnalis*. Negative results in OECD TG 242 do not necessarily mean that the chemical is

not a potential ED – a judgement about the endocrine disruption potential in other arthropods will have to be made based on a weight of evidence evaluation of existing *in vitro* and *in vivo* data.

References

Duft, M. et al. (2007), "Prosobranch snails as test organisms for the assessment of endocrine-active chemicals: An overview and a guideline proposal for a reproduction test with the freshwater mudsnail *Potamopyrgus antipodarum*", *Ecotoxicology*, Vol. 16/1, pp. 169-182, https://doi.org/10.1007/s10646-006-0106-0.

Li, J., M. Ma and Z. Wang (2008), "A two-hybrid yeast assay to quantify the effects of xenobiotics on retinoid X receptor-mediated gene expression", *Toxicology Letters*, Vol. 176/3, pp. 198-206, https://doi.org/10.1016/j.toxlet.2007.11.006.

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.2.4. ***Potamopyrgus antipodarum* Reproduction Test (OECD TG 242): 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.

Existing results: * "Mechanism (*in vitro* mechanistic data)" assumes that mechanistic data are available from available from suitable assays. Some assays concerning mechanisms of disruption in molluscs (e.g. retinoid X receptor [RXR] agonism) may be available, but they have not yet been internationally standardised. In practice, data from few if any assays may be available and therefore this must be taken into account when deciding on the "next step".

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 242	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 adverse *in vivo* effects in parthenogenetic molluscs, possibly but not necessarily caused by endocrine disruption, plus possible endocrine effects in other molluscs.	It would be desirable (if not already conducted) to perform an apical test with a sexually reproducing mollusc (e.g. the *Lymnaea stagnalis* Reproduction Test – OECD TG 243).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is endocrine active.
B	+	+	–	Strong evidence for adverse *in vivo* effects in parthenogenetic molluscs, possibly but not necessarily caused by endocrine disruption.	It would be desirable (if not already conducted) to perform an apical test with a sexually reproducing mollusc (e.g. the *Lymnaea stagnalis* Reproduction Test – OECD TG 243).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is endocrine active.
C	+	+	Eq/0	Strong evidence for adverse *in vivo* effects in parthenogenetic molluscs, possibly but not necessarily caused by endocrine disruption.	It would be desirable (if not already conducted) to perform an apical test with a sexually reproducing mollusc (e.g. the *Lymnaea stagnalis* Reproduction Test – OECD TG 243).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is endocrine active. 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	+	–	+	Strong evidence for adverse *in vivo* effects in parthenogenetic molluscs, possibly but not necessarily caused by endocrine disruption, plus possible endocrine effects in other molluscs.	It would be desirable (if not already conducted) to perform an apical test with a sexually reproducing mollusc (e.g. the *Lymnaea stagnalis* Reproduction Test – OECD TG 243).	The lack of *in vitro* evidence of endocrine activity is not evidence against any such activity, due to the limited nature of current *in vitro* screens.
E	+	–	–	Strong evidence for adverse *in vivo* effects in parthenogenetic molluscs, possibly but not necessarily caused by endocrine disruption.	It would be desirable (if not already conducted) to perform an apical test with a sexually reproducing mollusc (e.g. the *Lymnaea stagnalis* Reproduction Test – OECD TG 243).	The lack of *in vitro* evidence of endocrine activity is not evidence against any such activity, due to the limited nature of current *in vitro* screens.
F	+	–	Eq/0	Strong evidence for adverse *in vivo* effects in parthenogenetic molluscs, possibly but not necessarily caused by endocrine disruption.	It would be desirable (if not already conducted) to perform an apical test with a sexually reproducing mollusc (e.g. the *Lymnaea stagnalis* Reproduction Test – OECD TG 243).	The lack of *in vitro* evidence of endocrine activity is not evidence against any such activity, due to the limited nature of current *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.

Scenarios	Result of OECD TG 242	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	+	Strong evidence for adverse *in vivo* effects in parthenogenetic molluscs, possibly but not necessarily caused by endocrine disruption, plus possible endocrine effects in other molluscs.	Given the absence or equivocal nature of existing *in vitro* data, it would be desirable to obtain further *in vitro* data on endocrine activity if possible. It might also be sensible to conduct a *Lymnaea stagnalis* Reproduction Test (OECD TG 243).	If a new *in vitro* mechanistic assay is conducted, note that a negative result does not mean that the test material has no endocrine 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	–	Strong evidence for adverse *in vivo* effects in parthenogenetic molluscs, possibly but not necessarily caused by endocrine disruption.	Given the absence or equivocal nature of the *in vitro* mechanistic data, it might also be helpful to conduct an *in vitro* screen for endocrine activity. It might also be sensible to conduct a *Lymnaea stagnalis* Reproduction Test (OECD TG 243).	If a new *in vitro* mechanistic assay is conducted, note that a negative result does not mean that the test material has no endocrine 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	Strong evidence for adverse *in vivo* effects in parthenogenetic molluscs, possibly but not necessarily caused by endocrine disruption.	Given the absence or equivocal nature of the *in vitro* mechanistic data, it might also be helpful to conduct an *in vitro* screen for endocrine activity. It might also be sensible to conduct a *Lymnaea stagnalis* Reproduction Test (OECD TG 243).	If a new *in vitro* mechanistic assay is conducted, note that a negative result does not mean that the test material has no endocrine 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 is probably endocrine active without adverse effects in parthenogenetic molluscs, although it is possible that *P. antipodarum* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required. However, it might be desirable to obtain data from a *Lymnaea stagnalis* Reproduction Test (OECD TG 243) if these are not already available.	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is endocrine active.
K	–	+	–	The test chemical is probably endocrine active without adverse effects in molluscs, although it is possible that *P. antipodarum* responds atypically in this case.	If there is no activity in parthenogenetic or sexually reproducing molluscs, 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 endocrine active.

Scenarios	Result of OECD TG 242	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 is probably endocrine active without adverse effects in molluscs, although it is possible that *P. antipodarum* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required, but if data from a sexually reproducing mollusc species are absent, it might be desirable to conduct a *Lymnaea stagnalis* Reproduction Test (OECD TG 243).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is endocrine active. 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 is probably endocrine active without adverse effects in parthenogenetic molluscs, although it is possible that *P. antipodarum* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required. However, it might be desirable to obtain data from a *Lymnaea stagnalis* Reproduction Test (OECD TG 243).	The lack of *in vitro* endocrine activity is not evidence against any such activity, due to the limited nature of current *in vitro* screens.
N	–	–	–	The test chemical is probably without activity in molluscs.	No further action is necessary.	–
O	–	–	Eq/0	The test chemical is probably without activity in molluscs.	Some regulatory authorities may conclude that no further evidence is required. However, it might be desirable to obtain data from a sexually reproducing mollusc (e.g. the *Lymnaea stagnalis* Reproduction Test – OECD TG 243).	The lack of *in vitro* endocrine activity is not evidence against any such activity, due to the limited nature of current *in vitro* endocrine 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 is probably without activity in parthenogenetic molluscs, although it is possible that *P. antipodarum* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required. Also, if clear *in vitro* mechanistic data are missing, it might be desirable to obtain some.	If a new *in vitro* mechanistic assay is conducted, note that a negative result does not mean that the test material has no endocrine 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.
Q	–	Eq/0	–	The test chemical is probably without endocrine activity in molluscs.	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.

Scenarios	Result of OECD TG 242	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 test chemical is probably without endocrine activity in molluscs.	Some regulatory authorities may conclude that no further evidence is required. However, it might be desirable to obtain data from a *Lymnaea stagnalis* Reproduction Test (OECD TG 243) if these are not already available.	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.