C.2.6. Chironomid Toxicity Test Using Spiked Sediment (OECD TG 218) or Spiked Water (OECD TG 219)

Status: Assay validated by the OECD.

358. Modality detected/endpoints: This medium-term *in vivo* assay with the dipteran insect *Chironomus* spp. is responsive to juvenile hormone (JH) (ant)agonists and ecdysteroid (Ec) (ant)agonists which can interfere with such processes as metamorphosis, moulting and growth (e.g. Hahn, Liess and Schulz [2001]; Taenzler et al. [2007]; Jungmann et al. [2009]; Tassou and Schulz [2009, 2013]). It exposes the test organisms over a single generation. It is important to note, however, that none of the endpoints in this apical test are specifically responsive to JH- or Ec-active chemicals, and the assay will give positive results with many other substances. The lack of internationally validated mechanistic assays for endocrine activity in insects may prevent firm conclusions about whether test chemicals are endocrine disrupters (EDs) in this taxon, although *in vitro* assays for JH and estrogen (E) activity are available in the literature. However, the data from the test may nevertheless be of value for classification and hazard identification/characterisation.

Background to the assay

359. This assay can be run with one of several chironomid species, including *Chironomus riparius*, *C. dilutus* and *C. yoshimatsui*. It can also be operated in one of two formats, with the test chemical spiked either into the ambient water (OECD TG 219) or into the sediment (OECD TG 218), thus allowing sparingly soluble or hydrophobic chemicals to be tested. The test with *C. riparius* and *C. yoshimatsui* takes 20-28 days, while with *C. dilutus* it continues for 28-65 days. The exposure to a range of test concentrations begins with first instar larvae and continues to their fully emerged adulthood. The main endpoints are time to emergence, and emergence itself, but larval survival and growth may also be measured.

360. Available data from a two-generation test with *C. riparius* (Tassou and Schulz, 2009) show that an agonist of the JH pathway will impact emergence rate in a single generation, though the no-observed-effect-concentration (NOEC) becomes lower when considering the second generation. It is expected that chemicals affecting the Ec pathway will also have an effect on emergence.

When/why the assay may be used

361. Although OECD TG 218/219 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 JH or E activity of a chemical. Given the significant degree of endocrine system conservation across the arthropods, effects in OECD TG 218/219 may also indicate the possibility of related activity in other arthropods such as crustaceans (cladocera, copepods and decapods). However, many chemicals with non-endocrine action will also give positive responses in OECD TG 218/219.

362. It is not recommended that OECD TG 218/219 be deployed as a primary screen for JH or Ec activity and effects because of its lack of specificity, but it should be noted that there are no standardised *in vitro* screens for JH- or Ec- (ant)agonists, although some are described in the scientific literature (e.g. Dinan et al. [2001]; Smagghe et al. [2003]; Swevers et al. [2003]).

363. 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

364. Existing information on endocrine-related effects from other arthropods should also be considered before deployment of OECD TG 218/219, given the commonality of endocrine mechanisms in these taxa. Existing data available might also include one or more of a range of *in silico* or *in vitro* results which suggest that JH or Ec disruption may occur *in vivo* (but note the limitations of this approach, as indicated above). Such indicators of possible JH or Ec activity might include QSAR predictions of JH/Ec activity, "read-across" from *in vivo* results obtained with structurally related chemicals or positive results from an *in vitro* screen for JH/Ec (ant)agonist activity. In addition, *in vivo* data may also be available from one or more short-medium term assays, including the Short-Term Juvenile Hormone Activity Screening Assay (SJHASA), or the *Daphnia magna* Reproduction Test with male neonate option (OECD TG 211).

Scenarios: Positive and negative results combined with existing data

365. The scenarios (A to R) presented in Table C.2.6 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.

366. Positive results obtained with OECD TG 218/219 (Table C.2.6, Scenarios A-I) result in the conclusion that the test chemical has adverse apical effects, at least in insects, but these are not necessarily caused by JH or Ec activity. However, although a positive response of OECD TG 218/219 indicates that the chemical has adverse effects in insects, it should be noted that crustacean species such as *Daphnia* have a parthenogenetic reproductive strategy and so may respond differently to *Chironomus*. Therefore, if countries need further evidence concerning growth and sexual development, etc. in this phylum, a *Daphnia* Multigeneration Test (DMGT) and/or a Harpacticoid Copepod Development and Reproduction Test (OECD GD 201) would be able to provide information on adverse effects in other arthropod groups. In other words, in order to strengthen weight of evidence, a positive result in OECD TG 218/219 could be followed by OECD GD 201 (Level 4), and/or the DMGT (Level 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.

367. The situation in which OECD TG 218/219 gives a negative result (Table C.2.6, 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 218/219 is simply insufficiently sensitive.

368. If OECD TG 218/219 and existing *in vivo* data are all negative, but *in vitro* data reveal some JH or Ec activity (Scenario K), the probability is that the test chemical is not sufficiently potent to produce JH/Ec (ant)agonism *in vivo* in arthropods, or it may be rapidly metabolised. In such a situation, further testing is probably not necessary.

369. On the other hand, if OECD TG 218/219 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 insect, the chemical is possibly not a JH or Ec (ant)agonist acting in insects, but it may be more potent in species (e.g. crustaceans) 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 GD 201).

370. Finally, a negative OECD TG 218/219, set against a background of negative *in vitro* and *in vivo* data (Scenario N), suggests that the test chemical is probably not a JH or Ec (ant)agonist *in vitro* or *in vivo*, and further action is unnecessary.

371. 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 218/219, and this is reflected in Table C.2.6. However, a lack of mechanistic data on JH or Ec activity should ideally be addressed before any further *in vivo* testing is finally conducted, although as indicated above, *in vitro* JH/Ec screens have not yet been internationally standardised. On the other hand, if OECD TG 218/219 is positive, further *in vivo* testing would generally be needed to quantify any adverse effects in crustaceans, 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. 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. JH or Ec agonistic and antagonistic) could, depending on dose, lead to a minimisation or abolition of adverse effects, while in others two different modes of action (MOA) could potentially reinforce effects on the OECD TG 218/219 endpoint. If multiple MOA are suspected, either from the existing results or based on

quantitative structure activity relationship (QSAR)/read-across/integrated approaches, this situation should be investigated further if needed for regulatory decision making.

372. The scenario in which the results of OECD TG 218/219 are themselves equivocal has not been dealt with in Table C.2.6, 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. If possible reasons for false negatives are suspected, OECD TG 218/219 could be repeated (e.g. conduct it at lower concentrations which avoid systemic toxicity).

373. In summary, positive results in OECD TG 218/219 indicate that a chemical has adverse effects in insects which may or may not be via JH or Ec (ant)agonism. This may need to be followed up with further apical testing with crustaceans. Negative results in OECD TG 218/219 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.

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Table C.2.6. **Chironomid Toxicity Test Using Spiked Sediment (OECD TG 218) or Spiked Water (OECD TG 219): 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 juvenile hormone-(JH) or ecdysteroid (Ec)-based assays. JH or Ec assays concerning mechanisms of disruption may be available, but they have not yet been internationally standardised. In practice, data from all assays may not 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 a JH or Ec disrupter.

Scenarios	Result of OECD TG 218/219	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 insects, possibly but not necessarily caused by juvenile hormone (JH) or ecdysteroid (Ec) (ant)agonists, plus possible JH or Ec effects in other arthropods.	It would be desirable (if not already conducted) to perform an apical test with crustaceans (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201; and/or the *Daphnia* Multigeneration Test [DMGT]).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH or Ec (ant)agonist. It should be noted that chironomids reproduce sexually and therefore may respond differently to a parthenogenetic species such as *Daphnia*. It should also be considered whether the full extent of effects in chironomids needs to be followed up with a Chironomid Life-Cycle Toxicity Test (OECD TG 233).
B	+	+	–	Strong evidence for adverse *in vivo* effects in insects, possibly but not necessarily caused by JH or Ec (ant)agonists.	It would be desirable (if not already conducted) to perform an apical test with crustaceans (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201; and/or the DMGT).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH or Ec (ant)agonist. It should be noted that chironomids reproduce sexually and therefore may respond differently to a parthenogenetic species such as *Daphnia*. It should also be considered whether the full extent of effects in chironomids needs to be followed up with a Chironomid Life-Cycle Toxicity Test (OECD TG 233).
C	+	+	Eq/0	Strong evidence for adverse *in vivo* effects in insects, possibly but not necessarily caused by JH or Ec (ant)agonists.	It would be desirable (if not already conducted) to perform an apical test with crustaceans (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201; and/or the DMGT).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH or Ec (ant)agonist. It should be noted that chironomids reproduce sexually and therefore may respond differently to a parthenogenetic species such as *Daphnia*. It should also be considered whether the full extent of effects in chironomids needs to be followed up with a Chironomid Life-Cycle Toxicity Test (OECD TG 233). 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 insects, possibly but not necessarily caused by JH or Ec (ant)agonists, plus possible JH or Ec effects in other arthropods.	It would be desirable (if not already conducted) to perform an apical test with crustaceans (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201; and/or the DMGT).	The lack of *in vitro* JH or Ec activity is not necessarily evidence against any JH/Ec activity, due to the limited nature of current *in vitro* JH and Ec screens. It should be noted that chironomids reproduce sexually and therefore may respond differently to a parthenogenetic species such as *Daphnia*.

						It should also be considered whether the full extent of effects in chironomids needs to be followed up with a Chironomid Life-Cycle Toxicity Test (OECD TG 233).

Scenarios	Result of OECD TG 218/219	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)**			
E	+	–	–	Some evidence for adverse *in vivo* effects in insects, possibly but not necessarily caused by JH or Ec (ant)agonists.	It would be desirable (if not already conducted) to perform an apical test with crustaceans (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201; and/or the DMGT).	The lack of *in vitro* JH or Ec activity is not necessarily evidence against any JH/Ec activity, due to the limited nature of current *in vitro* JH and Ec screens. It should be noted that chironomids reproduce sexually and therefore may respond differently to a parthenogenetic species such as *Daphnia*. It should also be considered whether the full extent of effects in chironomids needs to be followed up with a Chironomid Life-Cycle Toxicity Test (OECD TG 233).
F	+	–	Eq/0	Some evidence for adverse *in vivo* effects in insects, possibly but not necessarily caused by JH or Ec (ant)agonists.	Given the absence or equivocal nature of existing *in vivo* data, it might also be sensible to conduct a JH/Ec-responsive crustacean assay (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201) or a JH-responsive DMGT.	The lack of *in vitro* JH/Ec activity is not evidence against any JH/Ec activity, due to the limited nature of current *in vitro* JH/Ec screens. It should be noted that chironomids reproduce sexually and therefore may respond differently to a parthenogenetic species such as *Daphnia*. It should also be considered whether the full extent of effects in chironomids needs to be followed up with a Chironomid Life-Cycle Toxicity Test (OECD TG 233). 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	+	Strong evidence for adverse *in vivo* effects in insects, possibly but not necessarily caused by JH or Ec (ant)agonists, plus possible JH or E effects in other arthropods.	Given the absence or equivocal nature of existing *in vitro* data, it would be desirable to obtain further *in vitro* data on JH/Ec activity if possible. It might also be sensible to conduct a JH/Ec-responsive crustacean assay (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201) or a JH-responsive DMGT.	If a new *in vitro* mechanistic assay is conducted, note that a negative result does not mean that the test material has no JH/Ec activity. It should be noted that chironomids reproduce sexually and therefore may respond differently to a parthenogenetic species such as *Daphnia*. It should also be considered whether the full extent of effects in chironomids needs to be followed up with a Chironomid Life-Cycle Toxicity Test (OECD TG 233). 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 218/219	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)**			
H	+	Eq/0	–	Some evidence for adverse *in vivo* effects in insects, possibly but not necessarily caused by JH or Ec (ant)agonists.	Given the absence or equivocal nature of the *in vitro* mechanistic data, it might also be helpful to conduct an *in vitro* screen for JH/Ec activity. It might also be sensible to conduct a JH/Ec-responsive crustacean assay (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201) or a JH-responsive DMGT.	If a new *in vitro* mechanistic assay is conducted, note that a negative result does not mean that the test material has no JH/Ec activity. It should be noted that chironomids reproduce sexually and therefore may respond differently to a parthenogenetic species such as *Daphnia*. It should also be considered whether the full extent of effects in chironomids needs to be followed up with a Chironomid Life-Cycle Toxicity Test (OECD TG 233). 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	Some evidence for adverse *in vivo* effects in insects, possibly but not necessarily caused by JH or Ec (ant)agonists.	Given the absence or equivocal nature of the *in vitro* mechanistic data, it might also be helpful to conduct an *in vitro* screen for JH/Ec activity. It might also be sensible to conduct a JH/Ec-responsive crustacean assay (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201) or a JH-responsive DMGT.	If a new *in vitro* mechanistic assay is conducted, note that a negative result does not mean that the test material has no JH/Ec activity. It should be noted that chironomids reproduce sexually and therefore may respond differently to a parthenogenetic species such as *Daphnia*. It should also be considered whether the full extent of effects in chironomids needs to be followed up with a Chironomid Life-Cycle Toxicity Test (OECD TG 233). 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 a JH or Ec (ant)agonist without adverse effects in insects, although it is possible that *Chironomus* spp. 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 crustaceans (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201; or the DMGT) 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 a JH/Ec (ant)agonist. It should be noted that chironomids reproduce sexually and therefore may respond differently to a parthenogenetic species such as *Daphnia*.
K	–	+	–	The test chemical is probably a JH or Ec (ant)agonist without adverse effects in insects or other taxa, although it is possible that *Chironomus* responds atypically in this case.	If there is no activity in crustaceans or insects, further evidence is probably not needed.	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH/Ec (ant)agonist.

Scenarios	Result of OECD TG 218/219	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 a JH or Ec (ant)agonist without adverse effects in insects, although it is possible that *Chironomus* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required, but if crustacean data are absent, it might be desirable to conduct a Harpacticoid Copepod Development and Reproduction Test (OECD GD 201); or a DMGT.	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a JH/Ec agonist. It should be noted that chironomids reproduce sexually and therefore may respond differently to a parthenogenetic species such as *Daphnia*. 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 without JH or Ec activity in insects, although it is possible that *Chironomus* 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 crustaceans (e.g. Harpacticoid Copepod Development and Reproduction Test – OECD GD 201; or a DMGT) if these are not already available.	The lack of *in vitro* JH/Ec activity is not evidence against any JH activity, due to the limited nature of current *in vitro* JH screens. It should be noted that chironomids reproduce sexually and therefore may respond differently to a parthenogenetic species such as *Daphnia*.
N	–	–	–	The test chemical is probably without JH or Ec activity in arthropods.	No further action is necessary.	–
O	–	–	Eq/0	The test chemical is probably without JH or Ec activity in arthropods.	Some regulatory authorities may conclude that no further evidence is required. However, it might be desirable to obtain data from crustaceans (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201; or a DMGT) if these are not already available.	The lack of *in vitro* JH/Ec activity is not evidence against any JH activity, due to the limited nature of current *in vitro* JH screens. It should be noted that chironomids reproduce sexually and therefore may respond differently to a parthenogenetic species such as *Daphnia*. 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 JH or Ec activity in insects, although it is possible that *Chironomus* 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 JH/Ec activity. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.

Scenarios	Result of OECD TG 218/219	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
Q	–	Eq/0	–	The test chemical is probably without JH or Ec activity in arthropods.	No further action is necessary.	It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
R	–	Eq/0	Eq/0	The test chemical is probably without JH or Ec activity in insects and possibly crustaceans.	Some regulatory authorities may conclude that no further evidence is required. However, it might be desirable to obtain data from crustaceans (e.g. the Harpacticoid Copepod Development and Reproduction Test – OECD GD 201; or a DMGT) if these are not already available.	It should be noted that chironomids reproduce sexually and therefore may respond differently to a parthenogenetic species such as *Daphnia*. 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.