Organisation for Economic Co-operation and Development

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Adverse Outcome Pathway External Review Report

AOP150: Aryl hydrocarbon receptor activation leading to early life stage mortality, via reduced VEGF (Vascular Endothelial Growth Factor)

Short Title: AHR activation to ELS mortality, via VEGF

The title of the AOP was revised as a result of the review.

Original Title of the AOP: Aryl hydrocarbon receptor activation leading to embryolethality via cardiotoxicty

1. Introduction and background to specific AOP

Background

The project for development of the AOP 150: *Aryl hydrocarbon receptor activation leading to embryolethality via cardiotoxicty* sprung out of a broader project submitted to the AOPs Development Programme in 2012 (project 1.7) to develop *the Adverse Outcome Pathways for Sustained Activation of the Aryl Hydrocarbon Receptor leading to a Range of Species-Specific Effects* led by BIAC and Canada.

The initial proposal was revised to cover two individual AOPs that were accepted in the AOP Workplan in 2013. One of these two individual AOPs led by Canada, the *AOP for Aryl Hydrocarbon Receptor 1 Activation Leading to Developmental Abnormalities and Embryolethality in Birds* was additionally broken down in two smaller linear AOPs:

- AOP 150: Aryl hydrocarbon receptor activation leading to embryolethality via cardiotoxicity, and
- AOP 131: AhR activation leading to uroporphyria,

AOP150 has undergone an internal review and modifications in early 2017 (Internal review AOP 150). Based on these, the Extended Advisory Group for Molecular Screening and Toxicogenomics (EAGMST) agreed at its June 2017 meeting, that the AOP150 draft [snapshot of 04-12-2017 PDF] was ready for external expert review. In addition, EAGMST recommended that AOP150 is reviewed in parallel with AOP 21: *AhR Activation Leading to Early Life Stage Mortality*, with which it shares several common elements.

A joint scientific review panel (Annex1) for both, AOP21 and AOP150, was selected by an independent review manager in accordance with the Standard Operation Procedure (SOP) for Adverse Outcome Pathway Scientific Review (v.7 December 2017).
The review panel was charged with reviewing the scientific content of the draft AOP based on four charge questions (CQ) previously agreed by the EAGMST and outlined in the SOP:

CQ1. Scientific quality:

- Does the AOP incorporate the appropriate scientific literature?
- Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?

CQ2. Weight of evidence:

- Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified?

CQ3. Regulatory applicability:

- Considering the strength of evidence and current gaps / weaknesses, what would be the regulatory applicability of this AOP, in your opinion?

CQ4. Conclusion:

- What are your overall conclusions of the assessment of this AOP?

In addition the joint panel was asked to particularly consider whether the content of the individual abstracts for each these two similar AOPs represent a clear stand-alone guidance for the users.

The review was conducted during December 2017 and April 2018. Based on the initial responses to the charge questions (Annex 2) main issues (Section 2) were discussed at a teleconference on 9 March 2018 (Section 3). Based on the TC discussion (section 3.2), actions arising (section 3.3), and additional written discussion (Section 4), authors revised the AOP as outlined in section 3.3. Revisions were considered by reviewers before this report was finalised. As a result of the review the title of AOP 150 was revised. The new title of AOP21 is: Aryl hydrocarbon receptor activation leading to early life stage mortality, **via reduced VEGF[1].**

Introduction

This adverse outcome pathway AOP 150: *Aryl hydrocarbon receptor activation leading to embryolethality via cardiotoxicity* includes the description and assessment of the critical elements of the pathway initiated by sustained activation of the aryl hydrocarbon receptor (AhR) during early embryonic stages, leading to embryolethatlity via cardiotoxicity.

The Molecular Initiating Event (MIE) of this AOP (Figure 1) is the activation of AhR by exogenous high affinity ligands/stressors leading to its nuclear translocation and interaction with the aryl hydrocarbon receptor nuclear translocator (ARNT). Persistent AhR/ARNT dimerization, induced by the high affinity binding perturbs the tightly regulated crosstalk between ARNT and its key partner for normal development of the cardiovascular system, the hypoxia inducible factor alpha (HIF-1α).

Under normal hypoxic conditions of the embryonic development, the ARNT/HIF-1α transcription complex activates genes involved in angiogenesis, including the vascular endothelial growth factor (VEGF), which is crucial for normal development of the cardiovascular (CV) system.

[1] **Vascular Endothelial Growth Factor**

Therefore, specific to this AOP is the indirect downregulation of VEGF leading to reduced cardiomyocyte and endothelial cell proliferation, altered cardiovascular morphology, reduced cardiac output and ultimately to congestive heart failure and death of embryos, particularly evident in birds.

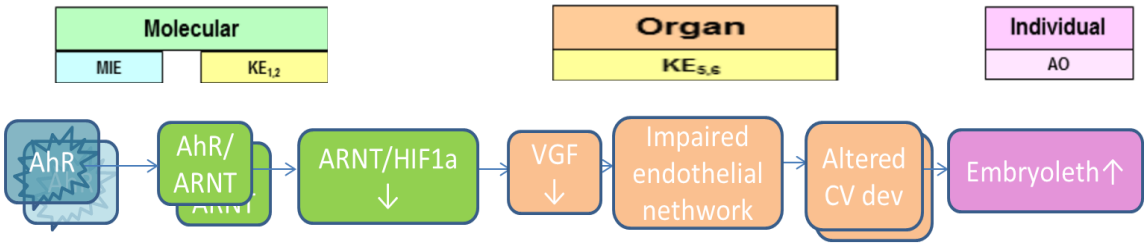

Figure1: Graphical representation of the components of AOP150. Duplicated elements are shared by AOP21 and also relate to other components of AOP21.

This pathway can be initiated by a range of planar aromatic hydrocarbons (PAHs) but most notable are halogenated aromatic hydrocarbons (HAHs).

The development of this AOP draws mostly on evidence from avian studies however, relevant evidence from fish and mammals is also included. Significant differences in sensitivity to stressors are evident between taxonomic groups.

Developing embryos of birds and fish are most sensitive to the stressors activating this AOP ultimately leading to embryos' death and population trajectory decline.

Mammals appear to be less sensitive. Early embryonic exposure to AhR-agonists in mice leads to cardiotoxicity that persists into adulthood, increasing susceptibility to heart disease, rather than embryolethality. However, in certain strains of rats it increased resorptions and late stage foetal death that was associated with oedema.

Quantitative correlation has been demonstrated between the AhR-binding affinity for the stressors and a reporter gene expression (indicative of AhR activation) with the AO in avian species. However, in non-avian taxa the differences in AhR binding affinity and how it relates to the sensitivity for this type of toxicity, is not investigated to the same extent.

AhR structure/isotype, binding affinity of the stressors and their metabolism and other cellular specific cofactors, may all contribute to the different sensitivity of taxa, life stage and cells in relation to this AOP. The AOP provides framework for elucidation of the mechanistic underpinnings of its differences. It also aims to provide a platform for chemical screening, ecological risk assessment and risk management of AhR agonists in relation to embryolethality in relevant species.

2. Synthesis of main issues of the review

Individual review comments are available in Annex 2 of this report.

The joint review of AOP 150 and AOP 21 presented a unique challenge for most reviewers. These two distinct adverse pathway share significant elements, some of which differ only in the life stage dimension (e.g. mortality at embryo or early adult stage), others in the taxonomic and even in the tissue applicability. All of this made the review demanding in terms of extracting specific issues for each particular pathway.

Few general issues were raised:

- Abstract and background should highlight the broader context of the interplay with AOP 21 and the distinct roles of COX2 and VEGF in embryo and cardiovascular development.
- Lack of clarity about the 'driving' component of AOP150 versus AOP21 following AhR activation by identical stressors. Related to this was also the title of the two AOPs and whether 'embryotoxicity' is clearly distinct from 'early life toxicity' at least in fish

It was suggested that a natural progression over time could be to combine the two AOPs. However, there was also a view that at present they represent two distinct AOPs.

An issue was raised regarding the 'action' for the protein dimerization activity in KE944: dimerization, AHR/ARNT being designated "disrupted" for both AhR and ARNT. While this may be appropriate for ARNT in AOP150 where the normal ARNT/ HIF1 is disrupted, it is not so for the AhR, particularly in AOP21 and AOP 131. KE944 is shared in all these AOPs.

Summary of responses to CQ 1 - Scientific Quality

There was a general agreement that the AOP150 incorporates the most important scientific literature and current scientific knowledge in this field.

Suggestions were made to:

- include additional references to help support the argument that AHR/ARNT/HIF interactions are conserved across taxa and better illustrate the crosstalk and interference between the AhR and hypoxia signalling pathways.

- better outline and reference the evidence for each stressor in the summary of stressor preceding the MIE (also raised in the context of CQ2)
- include references to relevant (Q)SAR approaches for predictive assessment of the MIE.

Summary of responses to CQ 2 - Weight of Evidence (WoE)

Reviewers agreed with the scoring of the WoE for the KEs and KERs.

In view of the joint review, the WoE for AOP150 was assessed as more convincing compared to that for AOP21. However, some reviewers found the tabular presentation of the Summary of WoE difficult to follow.

Summary of responses to CQ3 - Regulatory Applicability

Given the shared elements with AOP 21, it was remarked that, comprehensive set of AOPs covering cardiovascular (CV) alterations would be of great benefit for both, AOP150 and AOP21. In this context, additional consideration of the interconnectedness between COX-2 and VEGF mediated pathways, as well as the importance of other genes for cardiovascular development and function, would add value to the description of AOP150.

Reviewers view potential applicability of AOP150:

- to provide mechanistic information for development of testing strategies for AhR binding and activating substances relating to reproductive toxicity
- in supporting the use of toxic equivalency factor in regulatory risk assessment of mixtures of Dioxin Like Chemica DLC

Summary of responses to CQ4 - Overall conclusions of the assessment

Reviewers agree that AOP150 clearly presents the empirical evidences for a potential mechanism of AhR toxicity leading to early life stage mortality via cardiotoxicity. The assessment provides a solid overview of the biological plausibility, the strengths and uncertainties related to the KERs and the whole AOP.

Discussion of the links of this AOP to AOP21 and other CV toxicity relevant pathways was encouraged as part of the background.

Additional Question: Is the Abstract Section clear enough to stand alone from the AOP page

All reviewers suggested that the Abstract and Background sections can benefit from the inclusion of considerations of the cross talk with the related AOP21. Several specific edits were also suggested.

3. Summary record of the teleconference

9 March 2018, 3pm Paris time

Joint end-of-review teleconference (TC) was held for AOP1501 and AOP21. It was attended by all reviewers, the authors of the two AOPs and the review manager (Annex 1).

Before the TC authors provided initial written responses to most of the comments (Annex 2). These (as numbered in the Annex 2) provided the starting point for the discussion.

3.1. TC agenda

1. Introduction of participants

2. Short introduction by Review Manager (RM)

   - Context of the review process and the report content
   - Context of the guidance for development and assessment of AOPs
   - Joined overview of the AOP21 and AOP 150 with main issues

COMMON ISSUES for AOP21 and AOP150:

3. Need to **increase clarity** over the distinctive characters of the two AOPs (comm. no: 1, 2, 4 in both AOPs; comm. No: 25, 37 in AOP21; comm. No: 15a, 26, 27, 31, 32, 38 in AOP150)
   a) provide more context and discuss relevant aspects of the other AOP (comm. No: 31, 32 in AOP21; 15a, 16 in AOP150)
   b) consider the reference to early life mortality versus embryotoxicity in the AOP titles and as distinct aspects of the two AOPs (comm. No: 15b in AOP 150)
4. **KE944 (**dimerization, AHR/ARN**)** – AHR/ARNT dimerization: is action "decreased" appropriate? (comm. no: 3 for both AOPs)
5. **KE18 (**Activation, AhR**)**: inclusion of QSAR methods for predicting MIE and corrections within the description of current methods (comm. no: 9 in AOP21; no: 12 in AOP150)
6. **KER972** (Activation, AhR leads to dimerization, AHR/ARNT) - Quantitative understanding call: strong or weak with identical considerations? (introduced by RM)

7. Issues with **NCBI[2] links** (comm. No 7 AOP150)

SPECIFIC SCIENTIFIC ISSUES AOP21

8. **Inconsistences in taxonomic applicability (TA) discussion** (com. no: 7, 12, 13, 17, 19b, 27, 29)
9. Support for **KER1351**: KE2 (Cox2 induction) to KE3 (CV development/function) moderate or weak? (comm. No: 11a, 15) where particular issue is dealing with **KE2 essentiality** (comm. No: 11a, 19c, 30)
10. Add info about **KE442** (Decreased, Population trajectory)**, KER1490** (Altered, Cardiovascular development/function leads to Increased, Mortality) to support "strong" call (comm. no: 10b, 11b, 16, 18)
11. **KE1269** (Increase, COX-2 expression) – Detection methods for of COX2 protein (comm. no: 10a)
12. Overall clarity of the **WoE discussion** (com. No: 20, 19a)

SPECIFIC SCIENTIFIC ISSUES AOP150

13. **WoE summary tables**– (comm. No: 21)
14. Description of the **stressors in the AOP summary**, including strength of evidence (comm. No: 11, 18)
15. **KE945** (reduced dimerization, ARNT/HIF1-alpha) – **ontology term** 'decreased' for both components (comm. No: 13a)
16. **KE948** (reduced production, VEGF): detection assays (comm. No: 13b)
17. **KE110** (impairment, endothelial network): in vitro-to-in vivo extrapolation of data (comm. No: 15c)

OTHER

18. Abstract changes/additions to "stand alone"

19. Regulatory applicability/significance discussion for both AOP (Comm. No: 21-25 in AOP21; 23-27 in AOP150)

20. Overall conclusion about the AOPs – open discussion guided by the initial written comments (AOP21- comm. No: 26-32; AOP150 – comm. No: 28-32; RM note 12)

3.2. Main issues and responses during the call

Agenda item 2: The review manager provided short overview of the OECD Review process and the expected outcomes. Shortlist of common and specific issues was also presented and agreed:

[2] National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/)

Common issues:

1. How to emphasise the distinctive character of each AOP up-front and clearly, while providing sufficient context.

2. Adding info to KEs and/or KERs

    - When not an original author
    - When obvious but authors are not experts

Specific scientific issues AOP21

3. Inconsistences in taxonomic applicability discussion

4. KER1351: KE2 (Cox induction) to KE3 (CV development/function) moderate or week?

    - particularly KE2 essentiality

5. Overall clarity of the WoE discussion

Specific scientific issues AOP150

6. Overall WoE summary tables versus narrative

The discussion followed the more detailed agenda (above) where individual comments (as numbered in Annex 2) were grouped around a common issue.

Agenda item 3: There was a general agreement that the scientific basis of both, AOP21 and AOP150 is solid. However, most find simultaneous navigation through the life stage and taxonomic applicability of the two AOPs sharing a number of KE and KERs, a unique challenge. In this context it was argued that there is a need to find a way to emphasise the **distinctive character of each AOP** up-front and clearly, **while** at the same **time providing a sufficient context** of the other AOP.

In addition, one reviewer commented that both, AOP21 and AOP150 focus on one single gene (Cox2 and VEGF, respectively) leading to the adverse outcome, without sufficient discussion of the wider context links to other important phase I and phase II enzymes genes (e.g. cyp1a1) and pathways.

In terms of the lack of clarity of **distinctive character/aspects** of the two AOPs, reviewers emphasised that the issue is not a matter of the content of the AOP elements or their particular sequence in each particular AOP, but that during the joint review it was hard to keep focus on what was the **key trigger**(s) for one or the other AOP while reading common elements.

It was recognised that these issues may have been augmented by the outline of the pdf file provided for review. Many reviewers found the outline of the AOPs within the Wiki much more logical and appreciated the ease of tracking.

Response: Authors of both AOPs agree that AhR activation induces a number of genes. However, it was pointed out that the evidence linking AhR activation to impairment of CV development/function via Cox2 was strong, and while other genes (e.g. Sox9b, Cyp1a) may play a role in modulating these AOPs, evidence from knock-out studies, does not link these genes to mortality as an endpoint.

To address the context issue, it was agreed to modify the Background to emphasise that the "low resolution" of the Cox2↑ to altered CV development/function is a consequence of the limited supporting evidence and does not exclude refinement in the future (Action 1 below).
The proposed modification aims to help direct the users to consider other relevant pathways before the networks are developed which should address the issue of context in the future.

In terms of **key triggers** for AOP21 versus AOP150 following AhR induction, authors argued that currently, there is no information that would help distinguish them. It depends on time point observed, tissue, species etc. However, it was agreed that the interaction/common elements of the two pathways should be discussed, even briefly, in each of the abstracts (Action 2).

Reviewers also brought up the possibility that the lack of **clarity/distinction between the AO** embryolethality in AOP150 and the AO mortality applicable to two life stages, embryo and early life in AOP21, may also be part of the confusion. It was agreed by the authors to develop a common AO at the individual level (Action 3).

Agenda item 4: A reviewer questioned the "decreased" action call for KE944: AHR/ARNT dimerization.

Response:
In their initial response the authors agreed that the action needs to be changed to "increased". There was also agreement on Action 4 by all at the TC (Action 4).

Agenda item 5: Modification required for KE18 (MIE) in terms of assays for detection were discussed and agreed at the TC leading to Action 5.

Agenda item 6: It was noted that the call for the quantitative understanding (QA) of KER972 differs for AOP21 and AOP150 even though the considerations in the text are the same.

KER972 in AOP	**Directness**	**Weight of Evidence**	**Quantitative Understanding**
150	**Directly leads to**	**Strong**	**Strong**
21	**Directly leads to**	**Strong**	**Week**

It became clear that calls can be made for KERs in an AOP specific manner where considerations can be added in the KER free text. In the case of AOP21 and AOP150, the considerations are the same, but AOP21 authors gave lower weight due to the mostly indirect evidence supporting this KER.

Response:
It was agreed that the strength of the indirect evidence warrants a moderate call for KER972 in both AOPs (Action 6).

It was also considered useful as a general practice to aim to present the argumentation for the WoE and QA calls in a manner more specifically referencing the guidance criteria (e.g. stating "based on….."), to avoid discrepancies resulting from inconsistent application of the criteria (Action 7).

Agenda item 7: It was noted that NCBI links sometime lead to blank pages and it was questioned whether that is a place holder for some information.

Response:
Authors think that NCBI links do not function when a general term is specified rather than a species (e.g. birds and not *Gallus galus*). However, everybody agreed that the option to have a general term for taxonomic applicability is useful, particularly for KE, KERs and AO at higher biological level. How to deal with NCBI links, applying and/or excluding taxonomic applicability for a KE, KER and overall AOP should be discussed further with the Wiki and KB developers team.

Agenda item 8: This agenda item was initiated by comments to AOP21 but lead to a wider discussion about how determining applicability is approached for KE, KERs and overall AOP historically and practically in AOP21 and AOP150.

Response:
It was agreed that taxonomic applicability for KEs, KERs and overall for the AOPs will be reviewed (Action 8) in both AOPs following the principle:

- Call applicability for KE or KER is based on the species in which evidence is generated (including negative calls where evidence exists demonstrating NON-applicability)
- If applicability to more general taxonomic group is specified (particularly at higher organisational level and AOP level), a qualifier/justification would be included to indicate that taxonomic applicability is likely or uncertain considering (un)known structural and/or functional differences/similarities between the species in the group.

Agenda item 9: Discussion applicable to AOP21 only

Agenda item 10: Discussion applicable to AOP21 only

Agenda item 11: Discussion applicable to AOP21 only

Agenda item 12: Discussion applicable to AOP21 only

Agenda item 13: The group discussed how to increase the clarity of the overall WoE discussion for AOP150 in a tabulated form without reiterating all the evidence from the KEs and the KERs.

As a way forward the reviewer who raised the issue offered to provide specific suggestions in written format (Action 10)

Agenda item 14: Reviewers requested more information about the AOP150 stressors described in the summary.

Response:
In their written response the author agreed to modify the stressor table in the summary and reference evidence for general group of stressors. At the TC, this was confirmed and accepted by reviewers as sufficient (Action 11).

Agenda item 15: One reviewer noted that the Key event component table for KE945: reduced dimerization, ARNT/HIF1-alpha, contains action "decreased" for both components that in fact interact with each other leading to increased (hetero)dimerization.

Process	Object	Action
protein dimerization activity	hypoxia-inducible factor 1-alpha	decreased
protein dimerization activity	aryl hydrocarbon receptor nuclear translocator	decreased

Response:
From the view point of the "normal" (in the absence of stressors) cellular partners for ARNT and HIF1α, dimerization, the action "decreased" was considered as most appropriate from the available ontology terms.

No change was required as reviewer also thought that the text describes the KE well, despite the initial confusion caused by the action ontology terms.

Agenda item 16: Reviewer suggested adding RT-PCR as a method for measuring the KE948 (reduced production, VEGF).

Response:
This event was not created by the AOP150 author who noted that the evidence for VEGF production used in AOP150 comes mostly from protein level measurement. However, it was also noted that KE component process included for this KE by the original author is specified as "gene expression".

It was agreed that it is appropriate to add "protein synthesis" as an ontology term for the KE component process, and RT-PCR as another method in the KE description, (Action 12).

Agenda item 17: A reviewer noted that for KE 110 (impairment of endothelial network) includes only *in vitro* measurement assays, and questioned whether sufficient information is provided to explain how would the data be interpreted at higher organisational levels, i.e. how could an *in vitro*-to-*in vivo* extrapolation be assessed.

Response:
It was agreed that the issue of *in vitro*-to-*in vivo* extrapolation is beyond the scope of description of the AOP.

Author also indicated that some *in vivo* evidence for impairment of endothelial network measurement could be added but that would not change the current calls for KE or KE relationship related to KE110 in AOP150. In addition, this KE was reused by the author of AOP150 and they have been unsuccessful in communicating with the original author to discuss revisions.

It was suggested that, in addition to the current AOP-KB etiquette (which puts the onus on the AOP developers reusing elements from the AOP-KB for ensuring the revision is not affecting the meaning of the original entry), additional guidelines/considerations are included in the guidance (e.g. time frame for response before revision without further consultation are considered acceptable).

Given all of the above, the group considered that adding more assays to measure KE110 although possible, it is not a priority at this stage of the development of the AOP.

Agenda item 18: Reviewers provided some general and specific comments for revisions in the Abstract to shape them better to "stand alone" in describing the particular AOP.

a) for AOP150:
   - add contextual information including links to Cox2 pathway (comm. No: 36-38)
   - specific edits (comm. No: 33-35)

Response:
Authors agreed to make the suggested changes (Action 13)

Agenda item 19: In terms for regulatory utility, the group agreed that both AOPs provide a good scoping document for KEs and corresponding screening level assays of AhR inducing toxicants that could lead to impairment in CV development/function and mortality.

Furthermore, given that taxonomic applicability predominantly covers fish and avian species, both, AOP21 and AOP150, could have greater regulatory significance in the context of environmental safety assessment.

Agenda item 20: As indicated in the initial review comments, it was agreed that AOP150 represents a solid description of AhR-induced early life mortality, which will be further improved with the revisions following this review.

It was also discussed whether the two AOPs could eventually be joined into one single branched AOP.

In relation to the above, authors agree that it is difficult to ascertain the relative contribution of the Cox2 versus VEGF (AOP150) pathway to AhR-induced impairment in CV development/function in oviparous species. Furthermore, they argued that having

two independent AOPs, linked through common elements rather than one branched AOP, is the best possible description considering the current knowledge. They pointed out that what is very unique to these AOPs is the strength of the WoE for the indirect relationship between the MIE and the AOs which makes the elements in between informative only in certain circumstances.

Reviewers agreed that having the two AOPs separately is appropriate representation for these AOPs.

3.3. Action list with responses from author

Action Item from TC	Response/revision
1. Update Background in each corresponding AOP to provide a bit more context and emphasise that AhR activation as a pleotropic (network) effect activating a number of genes while the AOP focuses on data strongly supporting the role of a particular element (Cox2 or NIF1α/VEGF) for CV development and early stage mortality. (COMPLETED)	• Second paragraph in Background changed from: "It has since become evident that TCDD[3], and other AHR agonists, disrupt the normal development and function of the heart." To: "It has since become evident that TCDD is a prototypical agonist of the AHR: a transcription factor that modulates the expression of a vast array of genes involved in endogenous development and physiological responses to exogenous chemicals (Denison et al. 2011)." • The closing sentence already indicates VEGF as a focus of this AOP.
2. Include brief discussion/reference in the abstract of each AOP to possible links and overlaps with the other AOP (150 or 21, as relevant). (COMPLETED)	• The following statement has been added to the abstract: "There are also multiple targets of AHR activation, such as the COX-2 signaling pathway, that could potentially interact." • AOP21 hyperlinked to this statement
3. Authors of both AOPs to work together to develop a single individual AO (e.g. early life stage mortality) for both AOP. Relevant life stage distinction for particular species may be added in the free text of the KE or the KER leading to the individual AO. (COMPLETED)	• KER 947 title changed from "Increase, Embryolethality" to "Increase, Early Life Stage Mortality" • Relevant text transferred from KE351 (increased, mortality") by Jon Doering • Applicable information for fish was transferred from KER351 (AHR activation→increased mortality) to KER984 (AHR activation→early life stage mortality) by Jon Doering
4. Change action for KE944: dimerization, AHR/ARNT from "decreased" to "increased". (COMPLETED)	• Action term in Key Event Component table has been changed from "disrupted" to "increased" for both entries (AHR protein dimerization activity and ARNT protein dimerization activity).

[3] Tetrachlorodibenzo-p-dioxin

5. Change content of "how is this event measured" for MIE (KE18) • Correct sentence: [Full-length AHR cDNAs are cloned into an expression vector along with a ~~luminescent~~ reporter gene construct (chimeric luciferase, P-lactamase or CAT reporter vectors containing the appropriate response elements for the gene of interest]. • Add in silico approaches supported with reference Hirano et al (2015) EST 49:3795; Bonati et al (2017) Curr Opin Toxicol 2: 42; Sovadinova et al (2006) ETC 25: 1291. (COMPLETED)	• The term "luminescent" has been deleted. • A brief section on in silico approaches has been added under "how is it measured or detected" in KE18: "In silico homology modeling of the ligand binding domain of the AHR in combination with molecular docking simulations can provide valuable insight into the transactivation-potential of a diverse array of AHR ligands. Such models have been developed for multiple AHR isoforms and ligands (high/low affinity, endogenous and synthetic, agonists and antagonists), and can accurately predict ligand potency based on their structure and physicochemical properties (Bonati et al 2017; Hirano et al 2015; Sovadinova et al 2006)."
6. Modify the quantitative understanding of KER972 to 'moderate' for both, AOP21 and AOP150. (COMPLETED)	• WoE for quantitative understanding of KER972 (AHR activation→AHR/ARNT dimerization) has been changed to "Moderate" in widget (KER table on main page of AOP). o This translates to an updated KER WoE strength on the KER page.
7. For any revisions in the WoE calls consider specifying the particular criteria aspects relevant. (COMPLETED)	• A statement has been added in the "Quantitative understanding" section of KER972: "Because ARNT is a necessary dimerization partner for the transcriptional activation of AHR, it can be assumed that AHR interaction with DREs correlates with AHR/ARNT dimerization, which provides some insight into the quantitative understanding of this key event relationship. However, it is not clear as to whether AHR interaction with DREs is directly proportional to AHR/ARNT dimerization. Therefore, the quantitative understanding of this link is based solely on indirect evidence." o Note: In order to keep this KER broadly applicable to other potential AOPs, specific mention of the WOE call was excluded (since the WOE call is AOP dependant).
8. Review all taxonomic applicability calls and the justifications for applicability to wider taxonomic groups following the principle discussed under agenda item 8. (COMPLETED)	• AOP150 main page: o Mouse WoE changed to "Low" o Rat WoE added as "Low" o The following statement was added to taxonomic applicability text "Therefore, this AOP is most strongly applicable to birds and fish. Although strong AHR agonists cause foetal mortality in mice and rats (Kawakami et al. 2005; Hassoun et al. 1997; Sparschu et al. 1970), cardiac malformation is rarely cited as a cause of death. It appears that AHR-mediated effects on cardiaovascular development in mammals more frequently lead to long-term functional deficiencies rather than foetal death." For the remaining KE and KER pages, only species with specific empirical evidence were included in the widget tables. In general, the lower level KEs explicitly state

	species in which the page is applicable to, whereas at higher levels of organisation (organ and above) state animal classes for which the page is applicable to (usually supported by review papers rather than specific studies).
9. Add protein measurement methods for detection of cox2 induction.	• Not applicable to AOP150
10. Reviewer 4 to provide written suggestions for improvement of WoE summary table in AOP150. (COMPLETED)	• The format of the WoE table has been modified slightly to remove ambiguity in structure. o Same number of columns throughout table o The content remained unchanged except for the identity of the KER. Rather than KE1→KE2 the KER page number and title is specified.
11. Add supporting info/references in the table of stressors in the AOP150 summary. (COMPLETED)	• "diobenzo-p-dioxin" replaced with "**polychlorinated dibenzodioxins**" in Stressor table and given an evidence term of "high" o Supporting notes and references added. • Supporting info and references added for "**Polychlorinated biphenyl**" and evidence term of "high" was added. • "Dibenzofuran" replaced with "**polychlorinated dibenzofuran**" and given an evidence term of "High" o Supporting notes and references added.
12. Modify KE948 to add "protein synthesis" as an ontology term for the KE component process, and RT-PCR as another method in the KE description. (COMPLETED)	• "protein synthesis" and "protein level" are not existing ontology terms. o Added the only relevant option: o Process = abnormal protein level o Object = vascular endothelial growth factor A o Action = decreased o NOTE: Uncertain about what type of action is more adequate; the intended meaning is "decreased protein level" • Western blot, immunohistochemistry and quantitative RT-PCR were added as methods of measurement of VEGF protein levels and gene expression, respectively, including supporting references.
13. Update Abstract to: • make reference to common elements with the other AOP • clarify the reference to endogenous AhR functions related to development in the abstract and on page 40 of the AOP150 (comment number 35) (COMPLETED)	• First point addressed in response to action item # 2 • Wording of opening sentence on AOP has been modified as per the reviewers' recommendations: "Interference with endogenous developmental functions of the aryl hydrocarbon receptor (AHR) by sustained exogenous activation causes structural, molecular and functional cardiac abnormalities and altered heart physiology in avian, mammalian and piscine embryos" → "Interference with endogenous developmental processes that are regulated by the aryl hydrocarbon receptor (AHR), through sustained exogenous activation, causes molecular, structural, and functional cardiac abnormalities ~~and altered heart physiology~~ in avian, mammalian and piscine

	embryos" • The opening sentence under the "Key event relationship description" section in KER984 (page 40 in snapshot) now explicitly states what "endogenous function" the KER refers to: "The aryl hydrocarbon receptor is commonly known for its involvement in xenobiotic metabolism and clearance, but it also regulates a number of endogenous processes including angiogenesis, immune responses, neuronal processes, metabolism, and development of numerous organ systems"
Comment # (Annex 2)	**Response/revision**
10. The cross talk between two nuclear receptors is mentioned but which nuclear receptors the author meant? This is not clear to me. Technically, AhR is not a member of the nuclear receptor superfamily, but shares many of the same attributes and we can call it a ligand-dependent nuclear receptor. But what about the other nuclear receptor? AhR, ARNT, and (HIF-1α) are heterodimeric transcription factors belonging to the family of bHLH/PAS proteins. Did the author mean the crosstalk between AhR and hypoxia signaling pathways? (COMPLETED)	• The term "nuclear receptors" has been changed to "signaling pathways (AHR and HIF-1α)" in the abstract.
14a. KER973 – The author may consider including a nice review on crosstalk and interference between the AhR and hypoxia signaling pathways – Vorrink and Domann, Chem Biol Interact. 2014 July 25; 0: 82–88. (COMPLETED)	• This citation has been added to KER973 in empirical support of the relationship as well as in the inconsistencies section. • The only change to the text of the KER page is the addition of "Vorrink et al (2014b) provides a thorough summary of supporting evidence as well as contradictions and uncertainties in the literature." In the empirical support section. Reference Fleming et al (2009) (also suggested in comment no: 6 in annex 2) was considered but was not included. Although it supports the presence of cross-talk, it shows the AHR pathway being affected by hypoxia but not vice versa. The minimal effect of AHR agonists on hypoxia reporter was not reversed by ARNT over-expression, therefore it suggests an alternate pathway.
33. In the Background section, the sentence "Interestingly, AHR activation (by TCDD), inhibition, and knockdown …" references Wang et al. 2010 for this information. I could not find mention in Wang et al. 2010 (ToxSci 151(1) 225-237) to experiments using AhR inhibition or use of knockdowns. […] (COMPLETED)	• Wrong reference was included by accident. It has been corrected to Wang et al. 2013: Wang Q, Chen J, Ko C-I, Fan Y, Carreira V, Chen Y et al. Disruption of aryl hydrocarbon receptor homeostatic levels during embryonic stem cell differentiation alters expression of homeobox transcription factors that control cardiomyogenesis. Environ Health Persp. 2013; 121: 1334–43. doi: 10.1289/ehp.1307297

4. Further Discussion

Following the TC, a written discussion was continued regarding the implications of Action 3, (the development of new and common AO for both AOPs) for the titles and the indirect KER leading from the MIE to AO.

Authors developed a new AO: Early life stage mortality. Reviewers agreed that this is an appropriate revision.

Given that the two AOPs now share the MIE and the AO, the title for AOP150 was changed to "Aryl hydrocarbon receptor activation leading to early life stage mortality, **via reduced VEGF**"

Reviewers agreed that the new titles reflect well the AOP content and help distinguish clearly AOP150 from AOP21, whose title was changed to "Aryl hydrocarbon receptor activation leading to early life stage mortality, via increased COX-2"

In addition, the indirect KER984 in AOP150 that linked MIE to the AO in AOP150 is now modified and merged with the similar KER1492 from AOP21. Appropriate content (mostly related to fish) from KER1492 was added to KER 984.

During the TC the group also identified general AOP development points for further discussion by EAGMST, Wiki developers and the AOP training team:

- additional guidelines/considerations should be included in the User's handbook to facilitate necessary modifications by authors of new AOPs who use pre-existing KE and KER (discussion under agenda item 17).
- discuss and develop improvements for representing taxonomic applicability more clearly and consistently (see discussion under agenda item 7 and 8)
- consider adding new ontology term: decreased/increased protein level (related to agenda item 16 and action 12)

During reviewing of this report by AOP authors and reviewers, one author suggested consideration of the use of "cardiovascular toxicity" rather than "cardiotoxicty" across the AOP150 as evidence for VEGF in the establishment of early heart structure and anatomy seems less strong, while evidence for its role in angio- and vasculogenesis is clear.

5. Outcome of the external review

Initial review found that AOP150 represents a solid description of AhR induced early life mortality in birds and fish. Only few points of clarification were suggested which were addressed (see section 3.3) by the author before the draft review report was circulated to reviewers.

Interconnectedness of AOP150 and AOP21 was discussed at all stages of the review. Related revisions lead to AOP150 and AOP21 sharing additional common elements (see figure 2 below).

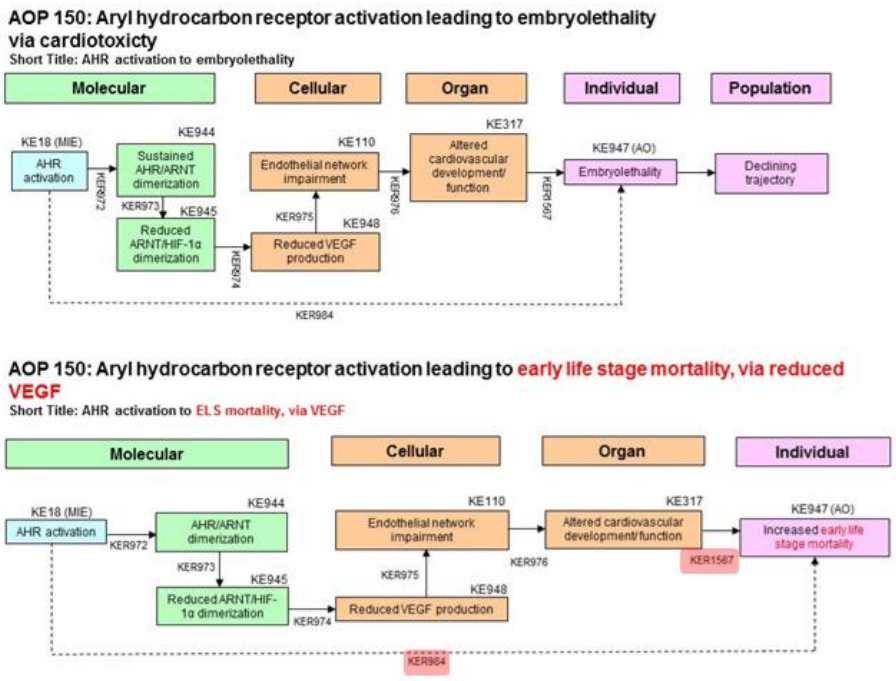

Figure2: Graphical representation of the components of AOP150 before and after revisions: Numbers represent KER numerical identifier in the Wiki. KERs and KE in red represent elements modified as a result of the review that now contain merged AOP21 and AOP150 content.

For AOP150 only the title of the AO at individual level was changed from "Embryolethality" to "Early life stage mortality". Related to this, the AOP150 title was

changed to “Aryl hydrocarbon receptor activation leading to early life stage mortality, **via reduced VEGF**”

All numbering of KE and KER pages remain unchanged in AOP150. Since the KE “declining trajectory” was never added into the wiki as a KE, it was removed from the graphical representation on the AOP150 Main page. The graphical representation is now the first item on the main page (rather than under “Overall Assessment of the AOP”).

The revised AOP150, will be a valuable addition to the AOP-KB. Together with AOP21, it provides a good scoping document for KEs and corresponding screening level assays for AhR inducing toxicants with potential impact on CV development/function and mortality, particularly in the context of environmental safety assessment.

Annex 1: List of Reviewers, Authors and Review manager

Expert name	Affiliation	Representing country
Michael W. Hornung	U.S. Environmental Protection Agency National Health and Environmental Effects Research Laboratory Duluth, Minnesota , USA	USA
Helen Håkansson	Institute of Environmental Medicine Karolinska Institutet Stockholm, Sweden	Sweden
Helmut Segner	Centre for Fish and Wildlife Health University of Bern Bern, Switzerland	Switzerland
Iva Sovadinova	Research Centre for Toxic Compounds in the Environment Faculty of Science Masaryk University Brno, Czech Republic	Czech Republic
Aude Kienzler	Joint Research Canter (JRC) Ispra, Italy	EC

Author	Affiliation
Amani Farhat	Environment and Climate Change Canada

Review Manager	Affiliation
Julija Filipovska	Independent Consultant

Annex 2: Individual reviewers' comments together with written response from the authors in preparation for the end of review Teleconference

Highlighted blue are comments identical for AOP21 and AOP150

General (relevant for both AOP 21 and AOP150)		Comment No	Written response from the authors
Reviewer 1	In general these did a good job of setting up the AOP and providing rationale and supporting evidence. I do think they stand alone as two distinct AOPs. I have suggested changes that can be made to the Abstracts and Background section to improve them.	1	
	I did find this review a unique challenge. Because there were significant sections that were shared components, at times I lost track of which one I was looking at. So I spent some time initially to determine what parts were specific to these AOPs and which parts were shared components pulled from the AOP Wiki.	2	
	Page 12 of both AOP21 and AOP150. 944: dimerization, AHR/ARNT (https://aopwiki.org/events/944); Short Name: dimerization, AHR/ARNT Why is the Action of the AHR/ARNT dimerization process classified as "disrupted"? For both the COX-2 and HIF1/VEGF pathways the dimerization is needed to get an adverse effect. It is required for the COX2 induction, and the AHR/ARNT dimerization is an essential step in removing ARNT from available cellular pools thereby reducing its availability to interact with HIF1a.	3	The KE components were added after the AOP was developed, so I don't know why this verb was used. I believe you are correct, so I have updated the action to "increased".
Reviewer 4	I think the scientific quality and analysis of the existing literature, the supporting evidence etc. is very well done in both AOPs – really a tremendous job. My main concern – and here I echo what the reviewer 1said – is the distinction of the two AOPs. It is not only that certain parts of the text are identical in both AOPs – why re-writing how the AhR activation works, it is the same in both pathways - but t is that I am afraid of the users can deal with the two AOPs. Assume I am a regulator and have a compound form which I know that it binds and activates AHR. Now I go to AOP WIKI and I find 21 and 150 – how to decide which one I should use for my compound? Both, only 21, only 150, or 150 in case of hypoxia and 21 in case of normoxia, or… I feel we have to give the readers something at hand to find their way	4	Yes, it would certainly be difficult to make sense of all the information on the AOPWiki for practical applications. This is why the AOPWiki is only one part of a larger Knowledge Base (https://aopkb.oecd.org/index.html), in which an AOP network (rather than a single path) is the functional unit. The interpretation of AOPs will be aided by a module called "AOP Xplorer"; it will create AOP networks and visual reports to simplify the mass of information. This Module is still under development, but relies heavily on the development of strong singular AOPs on the

			AOPWiki.
Charge Question 1: Scientific quality: Does the AOP incorporate the appropriate scientific literature? Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?			
Reviewer 1	Yes. The description of this AOP reflects the current scientific knowledge of the potential for the AHR to bind with ARNT, thereby reducing ARNT's availability to interact with other transcription factors such as HIF1 which is necessary for normal cardiovascular development.	5	
	p.17-19. Inclusion of a reference to Fleming et al here will help support the argument that AHR/ARNT/HIF interactions are conserved across taxa. (Fleming, C.R., Billiard, S. M., Di Giulio, R. T. (2009). Hypoxia inhibits induction of aryl hydrocarbon receptor activity in topminnow hepatocarcinoma cells in an ARNT-dependent manner. Biochemistry and Physiology, Part C 150, 383–389.)	6	Thank you, I will look at adding this as support.
	p. 19-20. KE 948: reduced production, VEGF (https://aopwiki.org/events/948). In the taxonomic applicability table, here and elsewhere in the AOP description, the NCBI links in which the last number is 0 take you to the NCBI Taxonomy Browser, but to a page that gives an error message: "Parameters error, no tax_id specified". It is unclear to me if these are just placeholder links in the current version.	7	I'm not sure why an NCBI link appears for such a broad category. The error likely occurs because it's not a single species that's specified, but an entire class of animal.
Reviewer 2	To my knowledge the scientific content of the AOP reflect the current scientific knowledge on the topic and I am not aware of a particular paper/review of importance that would have been forgotten.	8	
Reviewer 3	The current AOP is well written and reflects current scientific knowledge on this specific topic. The AOP diagram will be helpful. The abstract and background are well written, and both give a reasonable overview of the AOP.	9	
	Specific comments: **The cross talk** between two nuclear receptors is mentioned but which nuclear receptors the author meant? This is not clear to me. Technically, AhR is not a member of the nuclear receptor superfamily, but shares many of the same attributes and we can call it a ligand-dependent nuclear receptor. But what about the other nuclear receptor? AhR, ARNT, and (HIF-1α) are heterodimeric transcription factors belonging to the family of bHLH/PAS proteins. Did the author mean the crosstalk between AhR and hypoxia signaling pathways?	**10**	Yes, this is what is meant. Thank you for the clarification. The abstract will be modified to remove reference to receptor cross-talk.
	The stressors in the summary of the AOP should	**11**	General chemical categories

	include TCDD (not only general dibenzo-p-dioxin) as the prototypical AhR ligand. Which polychlorinated biphenyl does the author mean? The author should include an evidence for each stressor.		were included to avoid having to list all individual derivatives. I think it's better to be inclusive in this section. I agree however that the data on each stressor is lacking. I will add supporting references for the stressors, as well as explain broadly how halogen position on PCBs can affect their affinity for AHR.
	The MIE description (KE18) is clear and biologically plausible and is shared by four other AOPs in the AOP wiki. **Specific comments:** In reporter gene assays, P-lactamase- or CAT-based assays are not the luminescent reporter gene assays. The expression of P-lactamase is commonly measured using the fluorogenic P-lactamase substrates and the expression of CAT is measured radioactively or using a fluorescing derivative of chloramphenicol. But, for sure, more recently developed models used luciferase as a reporter gene with luminescent end-point. This MIE can be predicted and supported by in silico studies (SAR and QSAR methods) and the authors can consider involving some information on this topic and some references – for example Hirano et al (2015) EST 49:3795; Bonati et al (2017) Curr Opin Toxicol 2: 42; Sovadinova et al (2006) ETC 25: 1291.	**12**	Thank you for the clarification. So would simply removing the term luminescent suffice [Full-length AHR cDNAs are cloned into an expression vector along with a ~~luminescent~~ reporter gene construct (chimeric luciferase, P-lactamase or CAT reporter vectors containing the appropriate response elements for the gene of interest]. Or do you think the nature of each reporter should be specified? I will look into these references. Thank you.
	KEs and AO are generally well described. **Specific comments**: **KE945** - is the key of this AOP. It seems to be plausible, but could the cells compensate this lack of ARNT for other dimerization partners? Is the protein dimerization activity of ARNT also decreased? Which experimental data do support this? Additional explanation: I understand a concept of a reduced dimerization of ARNT/HIF1-alpha, but in the key event components are two components - 1. protein dimerization activity of hypoxia-inducible factor 1-alpha - decreased; 2. protein dimerization activity of aryl hydrocarbon receptor nuclear translocator - decreased. I have problems with that description. Which experimental data do support this decrease of protein dimerization activity of ARNT? And what about HIF1-alpha? Is there another dimerization partner for HIF1-alpha?	**13** **13a**	**KE945-** Most of the evidence provided shows the reduction in HIF1-a activity following AHR activation, or the reduction in TCDD toxicity following extreme hypoxia (references are on the KER 973: dimerization, AHR/ARNT leads to reduced dimerization, ARNT/HIF1-alpha page). The key event components are meant to describe the KE using structured ontology terms, to enable machine reading. The only available term for protein dimerization is "protein dimerization activity". I am not aware of any other

	KE948 – Can be measured the gene expression of VEGF-A using RT-PCR or Western blot? **KE110** – I recommend changing the level of biological organization from molecular to cellular.	**13b** **13c**	dimerization partners for HIF1-alpha. **KE948-** This page was meant to represent reduced protein levels, which is why PCR was left out as a method of detection. I now realize that the event component lists gene expression as the process. In this case RT-PCR would be applicable. Let's discuss which is more appropriate, and modify either the KE title (in which case we'd need permission from the developing author) or KE component, and subsequent detection methods. **KE110-** Agreed, and done.
	KEs and KERs are generally well described, explained and provide useful details to support the biological plausibility and the empirical support for linkage. **Specific comments:** **KER973** – The author may consider including a nice review on crosstalk and interference between the AhR and hypoxia signaling pathways – Vorrink and Domann, Chem Biol Interact. 2014 July 25; 0: 82–88.	**14** **14a**	Thank you. I will check it out.
Reviewer 4	Yes. The literature evaluation and the scientific quality are excellent. A critical point to me - and this point is not a matter of scientific quality, but a principal question – is the discrimination between AOP 21 and AOP 150. Both AOps share stressors – TCDD (AOP 150 additonally lists PCBs and dibenzofuran, AOP 21 lists PAHs) – and the same MIE. Both AOPs still share the first KE, AHR/ARNT dimerization, then however, they split up: wile AOP 21 moves to the KE "increased COX-2 expression", AOP 150 moves to "reduced ARNT/HIF-1 dimerization" and later to "VEGF". Mechanistically, this is clear and well explained in the text, however, the reader being nor particularly with the field may ask when does the MIE develop into the VGEF direction and when in the COX direction. In other words: If a reader has an AhR-binding compound, what criteria could he use to decide whether this compound will lead to embryotoxicity through the COX (AOP 21) or through the VGEF (AOP 150) pathway? Is it that the	15 15a	I appreciate your struggle with some of the AOP wiki's conceptual structures. The site has undergone multiple upgrades since its conception in response to questions such as these, and it will continue to improve with more constructive criticism. Regarding your first point, I will kindly refer you to my response on one regarding AOP KB and AOP Xplorer. Multiple efforts are underway by the developers to create interpretive tools based on networks and scoring criteria.

	AOP 150 will come into play "under conditions of hypoxia", as said in the text (and only then), while in non-hypoxic conditions the COX pathway comes into play? Here I would wish that the text gives more advise and information to the reader: two AOPs sharing the same MIE and basically the same adverse outcome, but taking different routes – when does which AOP work? I would find it extremely helpful for the reader to obtain some guidance on this. Related to this is a question on the title of the two AOPs: AOP 21 talks of AHR activation leading to "early life stage mortality" (what I interpret, at least for fish, embryos and larvae), whereas AOP 150 refers to "embryomortality". Is it indeed so, that the adverse activity of AOP is restricted to the embryo stage, while AOP 21 toxicity extends to the larvae? In the "overall assessment of the AOP, AOP 21 say "This AOP is only applicable starting form embryonic development..", and AOP 150 says "Exposure must occur early in embryo development.." what sounds fairly similar. Of course, this is much semantics, but I try to think from a reader's perspective who may get start thinking if there is meaning behind the different nomenclature of two so closely related AOPs. Otherwise, the description of how the KE work, how they are measured is done at a very good standard, under appropriate consideration of the literature. I have only one question concerning the KE "impairment, endothelial network" (maybe I missed the information in the text"): as a method for measuring tubulogenesis, an in vitro endothelial assay is described. How can effect concentrations measured in such an in vitro assay be transferred into the fish embryo system?	15b 15c	With respect to AOP 150, the AO is specific to early embryogenesis. Exposure late in development or early hatch will not lead to the same effects. I am not sure if this is the case for AOP 21, but based on the sentence you reference, it seems that it does not extend to the larval stage either. So maybe we could agree on a more consistent title for both AOPs following discussion. There are various computational methods for in-vitro to in-vivo extrapolation, but I am not well read on them. This KE was created by another user, so I'm not sure why in vivo methods weren't mentioned. On the main AOP page, in the essentiality section, there are a number of in vivo studies that potentially contain relevant methods. I have been unsuccessful in reaching the authors of this KE, and am not sure what the protocol should be for editing. Maybe Julija can advise?
Reviewer 5	Yes. The two AOPs #21 and #150 include appropriate information reflecting current knowledge on AhR activation, partnering with ARNT and other molecular events potentially preceeding functional cardiac consequences and lethality early in life. The selected focus on COX2 and VEGF, among the many genes involved in early cardiac development could be better motivated	16	

Charge Question 2: Weight of evidence: Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified?			
Reviewer 1	Overall the weight-of-evidence scoring is appropriate in this document. The section "Overall Assessment of the AOP" beginning on page 43 provides compelling evidence that this is a plausible AOP supported by experimental evidence.	17	
	Evidence scoring is missing in the Stressors box for the Summary of the AOP (page 2). The evidence could be listed as "Strong" for the three stressors. This table would need a footnote to indicate that the chemicals for which this is strong are those that fit the description of DLC chemicals. This should be clarified in a footnote, because not all dibenzo-p-dioxins, dibenzofurans, or polychlorinated biphenyls will produce these effects.	18	Agreed. More information will be included on each stressor category.
Reviewer 2	I globally agree with the weight-of-evidence scoring for KEs, KERs and the overall AOP, as well as with its applicability domain.	19	
Reviewer 3	Inconsistencies, uncertainties and level of confidence are provided for all KEs, KERs and the overall AOP. The level of support for essentiality of the KEs are adequately described and justified. The level of support for biological plausibility of each KER is reasonable and well justified. The overall weight of the AOP and the quantitative understanding are reasonable addressed.	20	
Reviewer 4	Simply for my understanding: in AOP 21, the WOE summary is presented in a descriptive way, going from plausibility through dose-response etc to consistency. In AOP 150, we have a descriptive part on the essentiality but then follows a tabular WOE summary (which by the way is difficult to understand) and then again "quantitative consideration". Any specific reasons for such differences in the presentation? Apart from such more formal things, I found the WOE discussion as presented to be convincing,	21	The tabular structure depicted in AOP150 was given as guidance for the authors in determining the WOE calls, so I thought it appropriate to include as a table. However, the coding to create a table on the Wiki was not straightforward and may be why some authors chose not to include it (it is much more simple now since the last upgrade). Only biological plausibility is included in AOP150. There are few studies in which measurements were made at multiple levels of organization, so dose and temporal concordance were not included. Why is the table difficult to understand? Can you suggest modifications for

			improvement?
Reviewer 5	The WOE discussion in #150 was more convincing to me as I knew beforehand about VEGF and its role in CV development and maintenance, while COX-2 connection (#21) to me was not that clear, and still is not, even though I did some literature review to become more familiar.	22	
Charge Question 3: Regulatory applicability: Considering the strength of evidence and current gaps / weaknesses, what would be the regulatory applicability of this AOP, in your opinion?			
Reviewer 1	This AOP provides a potential mechanistic explanation to bolster the regulatory application of toxic equivalency factors for AhR agonists for producing toxicity.	23	
Reviewer 2	This AOP gives a good mechanistic insight of a potential toxic pathway of AhR agonists and could help identifying the most sensitive species; it also supports the use of toxic equivalency factor in regulatory risk assessment of DLC mixtures.	24	
Reviewer 3	Reproductive/developmental toxicity is receiving increasing attention because of its adverse impact at the level of the species. Therefore, the European legislation REACH requires specific assessment of this type of toxicity. I see potential use this AOP in some integrative testing strategies or integrated approaches to testing assessment. In addition, this AOP has utility towards the mechanistic understanding of adverse effects of AhR agonists.	25	
Reviewer 4	My concern with the regulatory applicability of this and AOP and the AOP 21 is that regulators will have difficulties to decide when to apply which of the two AOPs.	26	True. I don't think the two are mutually exclusive, and likely occur simultaneously, so both should be considered in a risk assessment. Methods for the utility of AOPs in a regulatory contest are under develeopemnt.
Reviewer 5	From a regulators point of view I think these AOPs might become more helpful if they could include more information on links between the molecular and functional/organ/clinical levels. It is also important for regulators to understand relationship between COX2 and VEGF, as well as other genes of importance for cardiac development and function. What is each gene doing and when during embryo development. Meaning also, that for regulators it is likely difficult to decide on the use of #21 vs #150. Is the #21 meant for fish, birds and #150 meant for mammals? Access to comprehensive AOPs on CV system is likely to be of high importance for regulators as well as other professionals as such information is largely missing.	27	Both AOPs should be considered by the regulator, as they likely occur simultaneously; they simply represent different paths to the same end point. Both AOPs are most relevant to fish and birds. In fact, COX2 is mentioned in AOP150 as an alternate pathway (among others). Methods for the utility of AOPs in a regulatory contest are under develeopemnt, and include a "network view" that

			will help to identify the most important KEs considering all related AOPs (AOP Xplorer).
Conclusion: What are your overall conclusions of the assessment of this AOP?			
Reviewer 1	This AOP provides strong evidence that one potential mechanism by which AhR agonists can cause embryo- and early life stage mortality is via competition for ARNT, thereby preventing ARNT from heterodimerization with proteins such as HIF1, which are critical for normal cardiovascular development and function. The resultant mortality is a result of, or strongly contributed to, by this cardiovascular insufficiency. The linkages between the key events in this AOP follow logically and are supported by empirical evidence.	28	
Reviewer 2	This AOP illustrates a potential mechanism of AhR toxicity leading to early life stage mortality via cardiotoxicity. Empirical evidences are clearly reported. The assessment of the AOP provides a good overview of the biological plausibility, the strengths and uncertainties related to the KERs and the whole AOP.	29	
Reviewer 3	The overall assessment of the AOP is solid. The AOP is very well developed and detailed and useful information with reasonable of weight of evidence are provided.	30	
Reviewer 4	A very interesting and well documented AOP. The only problematic point is the linkage/separation to AOP 21 (what applies vice versa for AOP 21).	31	
Reviewer 5	The two AOPs provide up-to-date and detailed information on AhR-mediated cardiac toxicity with focus on COX2 and VEGF, respectively; two selected genes that are important during heart development. A broader context for how these genes play roles during embryo-cardiac development would be welcome and could be part of the background section. How do COX2 and VEGF interact with other important genes during this time window, and how are these genes regulated not only through AhR but also through e.g. the retinoid system, which also is a well-known vital regulator of the cardiac system during embryo development . A natural progression over time could be to combine the two AOPs, while at the current stage it would be helpful to explain and discuss possible links and overlaps between the two AOPs, as there are many commonalities.	32	We definitely agree that viewing an AOP in the contest of related biological processes is important, which is why the development and potential interpretation of AOP networks is underway. Standing alone however, an AOP is meant to describe <u>one</u> potential pathway from an MIE to an AO.
Additional question: The Abstract section of an AOP should, provide a concise and informative summation of the AOP under development that can stand-alone from the AOP page. Please consider whether all specific or important points for the AOP have been reflected in the Abstract so as to allow a user to decide on the suitability/applicability of one or the other (or			

both) AOP in their circumstances.			
Revie wer 1	**Abstract:** The abstract adequately describes this AOP and the specific KEs such that it is distinct and separate from similar AOPs with which it shares an MIE. This AOP describes the effect of AHR-activation which leads to a reduction in the available transcription factor ARNT for other normal cardiovascular developmental processes that require this hetero-dimerization partner protein. I would suggest a simple edit to the first sentence of the abstract to address this (see below). This change indicates it is not the functions of AHR that are being interfered with, but the sustained activation of AHR is what leads to interference with other developmental functions. "Interference with endogenous developmental processes functions of the aryl hydrocarbon receptor (AHR) by sustained exogenous activation of the aryl hydrocarbon receptor (AHR) causes structural, molecular, and functional cardiac abnormalities…"	**33**	I see what you mean, but I'm not sure I understand the term "process functions"; would simply replacing "functions" with "processes" suffice? "Interference with endogenous developmental processes of the aryl hydrocarbon receptor (AHR) by sustained exogenous activation causes […]"
	Background: In the Background section, the sentence "Interestingly, AHR activation (by TCDD), inhibition, and knockdown …" references Wang et al. 2010 for this information. I could not find mention in Wang et al. 2010 (ToxSci 151(1) 225-237) to experiments using AhR inhibition or use of knockdowns. They suggest that other cellular components of cardiovascular development such as a cardiac-specific homeobox gene, cardiac-specific troponin, α- and ß-myosin heavy chain are affected by TCDD thereby altering normal cardiomyocyte development; so it is not specific to a HIF 1α/VEGF pathway and thus may not be a strong supporting citation for a HIF1/ARNT/VEGF AOP. It is also unclear what is being referred to at the end of this sentence where it states "… indicating that AHR also has an optimal window of expression for normal cardiogenesis." Were there other studies to be cited here? This sentence should either be rewritten to clarify the points the author wishes to convey here, or alternatively this sentence could be omitted.	**34**	Thank you for noticing this error. It seems there may be additional references missing to support the information; I will have to go back and find these. The point I was trying to express is that AHR activation and inhibition/knockout have similar developmental consequences…which is somewhat counterintuitive.
Revie wer 2	The **abstract** describes well this AOP and it specific KEs. It also covers the assessment of the AOP (biological plausibility, main uncertainties, quantitative understanding) which make it quite complete. It is nicely completed by the background information. I would rephrase the first sentence "Interference with endogenous developmental functions of the aryl hydrocarbon receptor (AHR) by sustained exogenous activation" which seems a bit weird; can we really say the AhR has developmental function?	35	I'm not sure I understand. The AHR plays a role in normal cardio-development, as indicated by knockout models. Is it the term "function" that doesn't make sense? Can you recommend an alternative?

Reviewer 3	As I mentioned in Question 1, the abstract well describes the overall AOP with all specific and important points. Both AOPs are greatly overlapping through the same MIE and some KEs. In addition, the COX2 pathway (the KE in the AOP21) is mentioned as an alternative pathway in the AOP150. Is there any possibility that COX-2 and VEGFA signalling pathways can crosstalk? If yes, can be AhR involved?	36	I haven't come across any evidence for crosstalk between the COX-2 and VEGFA pathways, so I wouldn't be comfortable commenting on it.
Reviewer 4	In my opinion, the current Abstract does not allow the reader to decide on the suitability/applicability of AOP 21 vs AOP 150 (see above).	37	
Reviewer 5	In my opinion, the current Abstracts do not allow the reader to decide on the suitability/applicability of AOP 21 vs AOP 150. There is not enough contextual information, in my opinion, in the current Abstract to allow the reader to decide on the suitability/applicability of AOP 21 vs AOP 150.	38	
Editorial			
Reviewer 1	Other note: Reference #75 is missing the volume number and pages.		It was originally accessed online pre-print. The print details have been added.
Reviewer 2	Title: via cardiotoxicity		Corrected, thank you.
	KE 948, P20, under the Sex Applicability table: VEGF proteins have been… and characterized		Corrected, thank you.
	KER 974, p33, Uncertainties, second bullet point: : There is also the potential		Corrected, thank you.
	Note: The KE "Pericardial edema" that has been suppressed after the internal review still appears in the graphical representation of the online version of the AOP 150, this should be updated.		Updated, thank you.
Reviewer 3	Please, check through the AOP document for typos and misspelling.		
Reviewer 4	In my opinion, the current Abstract does not allow the reader to decide on the suitability/applicability of AOP 21 vs AOP 150 (see above).		