Scientific report for AOP 263 on Uncoupling of oxidative phosphorylation leading to growth inhibition via decreased cell proliferation

Reviewers comments and authors responses

Initial review

This review concerns the AOP publication authored by You Song and Daniel Villeneuve and submitted to Environmental Toxicology & Chemistry. The reviewed materials consisted of a snapshot of the AOP263 "Uncoupling of oxidative phosphorylation leading to growth inhibition," captured from the AOPwiki (https://aopwiki.org/aops/263) along with the accompanying manuscript titled "The adverse outcome pathway for uncoupling of oxidative phosphorylation leading to growth inhibition."

The four reviewers David Dreier, Ksenia Groh, Joel Meyer and Terry Schultz have jointly discussed, prepared, and approved the final review text below.

The reviewers commend the authors for the work carried out to prepare this submission. Uncoupling of oxidative phosphorylation (OXPHOS) is one of several important mechanisms that can lead to mitochondrial dysfunction and toxicity. The MIE "Decrease, Coupling of OXPHOS" is well characterized through multiple studies. The two KEs, "Decrease, Adenosine triphosphate pool" and "Decrease, Cell proliferation" are both at the cell/tissue level of biological organization. The AO "Decrease, Growth" can be assessed at various levels of biological organization, ranging from tissue to organism. Growth inhibition is an accepted regulatory endpoint, addressed, for example, by several OECD test guidelines (TG). In general, the reviewers agree with the AOP organization and description, as well as the assessments made by the authors with respect to the strength of evidence for individual KEs and KERs. However, several aspects, as listed below, might require further consideration and potential revision by the authors.

First, not only the uncoupling of the OXPHOS, but also several other mechanisms could lead to dissipation of the proton-motive force (PMF). Therefore, it may be important to understand whether the observed effects on the PMF are a direct consequence of uncoupling or secondary to another mechanism. In the light of this, we invite the reviewers to consider capturing the "dissipation of the PMF" as a separate KE in this AOP. Inclusion of this event as a separate KE entity could allow intersecting/linking with any future AOPs that would describe mechanisms other than OXPHOS uncoupling that could also lead to PMF dissipation; otherwise, these might remain disconnected.

We notice that the "Background" section in the AOP snapshot is rather short. Perhaps this was intentional, while the "Introduction and background" section of the accompanying manuscript captures more information. However, we feel that also in the manuscript, the readers would certainly benefit from the addition of several important references that are currently missing. In particular, we refer the authors to the following three publications:

- Ebert and Goss 2020: While this paper is purely mechanistic modeling to predict protonophoric uncoupling activity, it has an extensive list of references following the science of respiratory uncoupling. The authors should consider including some of these references in their overview.
- Schultz et al. 2002: This paper presents a comparison of pentachlorophenol (PCP) results with those elicited upon exposure to the model nonpolar narcotic 1-octanol, which revealed marked differences in both growth kinetics and the relative percentages of selected fatty acid methyl esters (FAMEs) in both pellicle and mitochondrial membranes.
- Hawliczek-Ignarski et al. 2017: This paper provides further evidence with regard to PCP's MoA. It is also a good example of how toxicogenomic data could be used to inform AOP development and which kind of testing data could be obtained with toxicogenomic approaches, so the

authors might also consider discussing this particular aspect as well, particularly in the section focused on the toxicity assays relevant for this AOP.

Overall, we do not suggest that the reference list of the presented manuscript needs to approach 100 or even 50 references, but the final reference list should both reflect the history of the subject and identify the key publications along the way. For example, consider also the contributions by Hanstein 1976, McLaughlin and Dilger 1980, and Mitchell 1966. Lastly, we observed that in many cases the references cited in the AOP snapshot are not included in the manuscript. We feel that most of the omitted references would make for a useful addition to the manuscript as well, especially considering that the manuscript might reach a wider readership and therefore needs to be more extensively supported by references, compared to the entry in the AOP wiki.

We also feel that the role and potential significance of the uncoupling proteins (UCPs) may be worth mentioning as well. UCPs are produced and regulated endogenously. Some UCPs play a role in heat generation, while others may play a role in modulating mitochondrial reactive oxygen species production (i.e., mild uncoupling, by decreasing the degree of reduction of ETC complexes, can decrease leakage of electrons to oxygen). See, for example, publication by Brand and Esteves 2005. We invite the authors to consider if the evidence on the effects of UCPs on some downstream events could further support the essentiality of those KEs?

Alternately, we see that down the road, the authors plan to connect uncoupling to mitochondrial ROS production. Inclusion of information on the connection of uncoupling to mtROS production may be more pertinent at that time. At this point, our understanding is that the relationship of mitochondrial uncoupling to mtROS production is that low levels of uncoupling decrease mtROS production, but high levels of uncoupling would eventually cause severe enough loss of mitochondrial homeostasis that mtROS increases. We suspect that this higher-"dose" effect may occur in the context of cytotoxicity, though, where mtROS production may be an effect rather than the proximate cause of mitochondrial dysfunction and cellular toxicity. However, a low-level decrease of mtROS levels could also be deleterious, especially in development, since mtROS signaling is important in developmental patterning and wound healing (Love et al. 2013; Timme-Laragy et al. 2018).

The potential for uncoupling to trigger a compensatory increase in glycolysis should perhaps be mentioned as well. This mechanism has been observed/known for a long time (Weinbach 1957) and it may actually bypass or reduce an apparent decrease in ATP (see for example Bestman et al. 2015), yet still result in an overall decrease in energy availability and growth since glycolysis is less efficient compared to OXPHOS. This important point may also need a separate discussion/mentioning in the section on the overall evidence assessment for this AOP and its KEs and KERs, as well as in the section that discusses alternative tests for this AOP. This is because there is also evidence that you can have compensatory upregulation of other energetic pathways, which will still come at a cost because this also requires energy. With this, you will not be observing an ATP decrease in vivo, although energy limitation would still be occurring, because of the overall less efficient use of available food resources. In vitro, if one would grow cells capable of glycolysis, uncouplers could appear much less toxic under these conditions than if the cells are forced to respire. See, for example, Marroquin et al. 2007.

Page 4 of the AOP snapshot: Table for the Essentiality of the Key Events says that "There are currently no inconsistencies and uncertainties identified by the authors." However, the authors themselves have cited, for example, the case when ATP pool increases upon mild exposure to uncouplers. While the

authors do offer an explanation for why this might be the case, should this not be considered a remaining uncertainty in this pathway, as long as the underlying mechanistic and quantitative relationships have not been characterized in more detail?

Concerning the mentioned "AOP network," of which the AOP 263 is said to be a "core" part of: We understand that the "AOP Network" is part of AOP-Wiki, but this mention is perceived as a detractor from AOP 263. With regard to this network, it is at the moment not clear, how much of it is still purely theoretical and how much is already listed and well-described with accompanying evidence collected and presented in the AOP wiki. Overall, we feel that, since the manuscript also repeatedly refers to the AOP network in the wiki, then it should also – at least briefly – explain the status of other AOPs belonging to the overall "network" of the AOP 263. Furthermore, the authors need to explain why the presented AOP 263 forms "the core of a larger AOP network" (as compared to the other AOPs in the network – which specific quality or descriptor makes it "the core" of the whole group?) We further observe that the network view contains connections to certain other forms of mitochondrial dysfunction (e.g., CIII and ATP synthase inhibition) but not others (e.g., CI, CII, CIV, redox cycling, Krebs cycle, etc.)—presumably, these will be added in the future? It would be helpful if the authors could comment on this as well.

Looking at the list of the AOPs which include the MIE "Decrease, Coupling of OXPHOS" (on page 9 of the AOP snapshot), one cannot help but wonder whether all 6 (!) AOPs with practically identical names are truly necessary (i.e., all are called "Uncoupling of oxidative phosphorylation leading to growth inhibition", with numbers 1-6 included in the end). We feel that the authors should provide a more detailed explanation, both in the snapshot and in the manuscript, as to why they find this granular structure necessary and what are the benefits they expect to gain from the proposed formulation of this particular "network" consisting of closely related if not nearly identical pathways.

We point out that event 1771 is also supported by two other studies, i.e., Luz, Lagido, et al. 2016 and Luz, Godebo, et al. 2016, which showed that *in vivo* exposure of *C. elegans* to FCCP caused an increase in oxygen consumption coupled to a decrease in steady-state, *in vivo* ATP levels.

With regard to the "overall assessment" for the KER3 of this AOP (event 1521): this event is supported by in vivo evidence from Bestman et al. 2015. This study has already been cited by the authors in support of the event 1821. However, the findings from this study are worth discussing with regard to event 1521 as well, because it reveals the unsurprising potential for cell- and tissue-specific effects to become larger when they are high-energy-use, potentially leading to teratogenesis in addition to growth inhibition (a mechanism that could perhaps form another AOP?). Therefore, the Bestman et al. 2015 reference should also be cited here as an in vivo example to make a point that there can be large tissue-specific effects and that not every cell type is equally susceptible. The authors should perhaps mention this as a placeholder, in order to ensure that the respective additional AOPs will at some point get constructed as well. These AOPs could also be seen as potential branching points to the AOP in question.

Overall, we do understand that a single AOP cannot be expected to capture all related evidence. However, we also feel that it is quite important to find the right balance between the understandable desire of the authors to be succinct and describe only what's necessary, but at the same point to avoid a situation when the 'naïve' people who would come and read this description would walk away with a feeling that growth inhibition is the only effect that uncouplers might lead to, or that growth inhibition is only caused by uncoupling. Therefore, we feel that more granularity in the descriptions for some KEs, as

well as some more details provided when discussing the supporting evidence, might make for a valuable addition to the manuscript. Our main concern is that we do not want this pathway to be interpreted in isolation and therefore we feel that the potential additional mechanisms, as well as some conflicting evidence, should be properly mentioned and discussed as well. As a suggestion, the authors could consider adding a sentence to their Discussion section in the manuscript, which should explain that, while this particular AOP is focused on a specific, necessarily limited chain of events only, it is also recognized that there are additional outcomes possible. Some of these additional outcomes should then be listed as examples, without being exhaustive of course. The goal of this addition would be that a reader which is new to the field would at least become aware of the associated complexity. This could also open the door to some other AOPs to be developed.

For example, the authors should discuss in more detail the link to teratogenesis as an outcome of growth disruptions manifested in certain organs. Indeed, the occurrence of both the malformed progeny as well as runts (smaller, often retarded siblings within one litter) are both caused by the effects related to developmental toxicity and dependent on cell proliferation capacity. Therefore, both can be seen as hazard endpoints that can be influenced by uncouplers. In the adult (mature) organisms, cell proliferation-related effects could also be particularly relevant for the tissues that maintain active proliferation status throughout life, e.g. gut which is always in a state of active turnover. In contrast, this certainly would not affect the brain, as you typically do not get more neurons. The latter point might again be true in mammals but much less so in fish, where neural tissue proliferation remains a life-long possibility. Some systemic effects (e.g., cardiac toxicity) also partially depend on cell proliferation. Overall, a better characterization of the AO at the organ level should capture some of this discussion. The authors should also add a sentence or two highlighting that there could be related outcomes other than organismal growth. Such discussion helping to relate the AO postulated in this AOP to some of the more traditional in vivo endpoints has the potential to further improve the presented AOP and its usefulness in the context of risk assessment.

Further, on page 3 of the AOP snapshot, in the section on the life stage applicability domain, the authors state that “Classical uncouplers such as 2,4-DNP have been reported to cause weight loss in adult humans […] suggesting that adults are partially in the applicability domain of this AOP.” This statement can and probably should be further strengthened. In fact, 2,4-DNP was sold legally for this purpose (i.e., weight loss), until its legal sale was banned because some people took too much of it and died as a result. This chemical, however, is still available online, and still killing people, unfortunately, see e.g. the report by Baker and Baker 2020. Therefore, human adults are indeed affected and susceptible to the effects of OXPHOS uncouplers.

The susceptibility of adult humans to mitochondrial uncoupling is further supported by what appears to be the first report of a (genetic) mitochondrial disease in people, namely the Luft Disease (Luft et al. 1962). This disease is thought to be caused by mitochondrial uncoupling (unfortunately, the specific gene(s) responsible for this mechanism remain unidentified) and is characterized by hyperthermia, perspiration, and enormous appetite despite low weight. The patient was underweight as a child, despite increased appetite. Again, this example further supports the idea that the uncoupler effects have high human health relevance.

Likely beyond the scope here, but perhaps worth keeping in mind as well: there is currently evidence of mitochondrial uncoupling leading to either increased or decreased neurodegeneration—perhaps related to the non-monotonic effects on mtROS. This could also be another AOP to be developed later.

Overall, we strongly emphasize that for this AOP and the associated effects, the environmental (ecotoxicological) and human health aspects should not be discussed in isolation. Currently, we observe a certain tendency of this AOP to lean more towards discussing the ecotoxicological aspects and applications, while the potential human health effects have been discussed rather cursorily and without going into much detail. We feel that there should be more discussion related not only to environmental health concerns but also to human health concerns, in order to better outline how these findings are specifically related to human health. We do understand that this AOP might have more of an ecotox flavor based on the authors' main expertise, but we do encourage them to expand it according to the directions suggested above.

On the other hand, with regard to ecotoxicological applications of this AOP, we were also somewhat surprised to observe that, while the authors do talk about growth on the tissue, organ and organismal level, they have not outlined any potential connection to population-level outcomes. At the same time, this AOP does place a lot of emphasis on its ecotoxicological relevance, as we have just discussed above. Therefore, we consider that it would be valuable if authors also compiled the evidence available with regard to potential population-level effects as well.

Further, with regard to environmental relevance, the authors should please elaborate on the significance of a lower acute-to-chronic ratio (ACR) for uncouplers (mentioned at line 255 in the manuscript). An important point to make here could be to explain, what the potential consequences of that could be.

With regard to the sex applicability domain of this AOP, we encourage the authors to consider including a study looking at PCP-caused decreased growth in rats (Schwetz et al. 1978), as there are also some sex-specific effects described in this paper.

The statement on Page 3 of the AOP snapshot, "The chemical applicability domain of the AOP mainly includes weak acids, such as …." is accurate, but perhaps it would also be helpful to explain why this is the case. That is, describe that uncouplers typically have properties as both weak acids and hydrophobic substances. As weak acids, they are capable of gaining and losing an electron. As hydrophobic substances, they are capable of distributing a negative charge over a number of atoms (often by π-orbitals which delocalize a proton's charge when it attaches to the molecule), so that they can diffuse back and forth across the IMM in either the charged or uncharged state, thus moving protons back across the concentration gradient generated by the ETC. A more detailed discussion of these mechanisms could be useful for any future analyses by scientists who might be interested to apply physicochemical property analysis to discovery of uncouplers.

We also observe that the chemical applicability domain of this AOP, which appears to be mainly focused on weak acids, might be unnecessarily narrow. It is not completely clear to us if hydrophobic ion or SH-reactive types of uncouplers have been considered/included as well. It would be helpful if the authors could clarify this point.

We further note that historically (e.g., in the cases of AOPs on skin sensitization or AOPs for estrogen-mimicking substances), AOPs have always included some discussion of applicability domains. However, one also needs experimental data on classic uncouplers within themselves to suggest an applicability domain. For example, 2,4-dinitro-, pentachloro- and 3,5-dichloro-phenol suggest the phenolic weak acid domain. But not all nitro/chlorophenols are uncouplers of sufficient strength to decrease growth before death occurs. In our view, one of the seminal functions of an AOP could be guiding direct testing to define the boundaries of its applicability domains.

With regard to the section on alternative assays: It is noted that three out of four KEs in this AOP can be measured using high-throughput in vitro assays. We were wondering if data from these assays could also be used as empirical support for the key event relationships? We suggest that, for assays that capture multiple key events, this information could be added to the concordance table, i.e. Table S1 in the supplementary material, as these would constitute useful additional lines of evidence for the key event relationships. For further information on the multiplexed assays, see Shah et al. 2016.

We also observe that a more detailed description of assays that could be used and would be important for the endpoints associated with the MIE and KEs in the outlined AOP is very critical, because this will go through the OECD. For applications there, it is not enough to just have a pathway, but you also need to have the assays with which it can be measured. Consequently, an AOP could be stuck at OECD if there are no good ways to measure an important KE. Therefore, better outlining these assays would be a critical point to move forward with this AOP.

AOP snapshot, page 2, the section on stressors: “moderate” evidence is given for pentachlorophenol while “high” is mentioned for all other listed chemicals. We were not able to locate a clear explanation for why the evidence for PCP is only moderate. In this regard, we also invite the authors to consider PCP-related evidence from studies by Schultz et al. 2002 and Hawliczek-Ignarski et al. 2017.

Further with regard to regulatory significance and potential applications of this AOP: We observe that the AO “Decrease, Growth” refers to growth inhibition, which is accepted as a regulatory endpoint in many countries (though not all) and has been addressed by several OECD test guidelines (TG). In a regulatory context, effects on growth can be measured with parameters such as length, wet or dry weight, or as a rate over time (as is common in algae). The authors do list some of these TGs in the section “Regulatory significance of the AO.” This section could be further expanded to also include guidelines for chronic toxicity testing in fish (TG 210) and birds (TG 206), thereby improving the applicability of this AOP as a framework for animal alternative approaches. In addition, the authors may consider adding a short discussion of the main differences in legislative mandates that some countries have with regard to growth as a regulatory endpoint. Overall, we feel that the discussion in this section should be expanded to explain how this AOP relates to the real world in terms of regulatory practice. For example, the authors could provide concrete examples linking different organisms to the listed TGs, as this would allow different regulatory bodies from across the world to better relate to this particular AOP.

While the ‘consideration for potential application of the AOP’ is optional in the AOP-Wiki, we deem it highly critical to publication in ET&C. We suggest that each of the presented considerations should be discussed in more detail in the manuscript. That is, not just listing with one sentence, but elaborating and presenting additional evidence and further considerations, as well as concrete examples or potential case studies for each point, where available.

With regard to the presented considerations themselves, we agree with most of them. One exception, however, is the fourth consideration, stating that the AOP is "highly generalized and has wide biological and stressor applicability domains, making it a central hub for many other AOPs." We understand that this consideration may stem from the assumptions and expectations associated with the previously mentioned "AOP network." However, we feel that this is rather speculative, as no specific proofs have been provided so far and we are not completely convinced of the utility or applications of this particular network (see also above for additional considerations regarding the "network" aspect).

We also suggest that the authors try to better illustrate the connections and interdependencies between the points raised. For example, linking considerations 3 and 5 should be emphasized, as this seems to be the classic way that the AOP provides the mechanistic/mode of action plausibility/probability needed to identify the most endpoint relevant and key event-related test systems, which, when used, could help define the boundaries of 2D structure applicability domains and establish structure alerts for predicting potency by read-across or QSAR.

One final consideration that came to our mind: can it be identified, which KE (or an MIE) represents the rate-limiting step in this AOP? This thinking was triggered by the estrogen-mimic AOP where ER-binding is the rate-determining step and fish liver vitellogenesis assay confirms this. The male-to-female gonadal conversion, feminization of male fish, and reproductive impairment are all downstream events that added weights-of-evidence to that AOP, but data for these events are not needed to make a regulatory decision. However, perhaps these considerations are going a step too far?

Thank you for providing the Tox21 data in the supplementary table S2. The assay documentation indicates this assay measures the mitochondrial membrane potential, and ATP content is used to measure cell viability in the assay (Attene-Ramos et al. 2015). If possible, it would be useful to include the cell viability data to discern specific effects on the mitochondrial membrane potential from general cytotoxicity. Providing both measures would give a clearer context for interpreting these data. Additionally, it is important to note this assay does not measure uncoupling directly, but rather, quantifies changes in the mitochondrial membrane potential as a potential consequence of uncoupling. Indeed, this information has been used to prioritize substances for additional mechanistic studies to identify uncouplers (Xia et al. 2018). It may also be important to note other high-throughput screening assays, such as respirometric screening assays, that can be used to identify specific mechanisms of action, including uncoupling (Hallinger et al. 2020).

<u>Minor comments</u>

In the sentence "A number of chemicals can bind to the inner mitochondrial membrane" (in the Background section), "bind to" should be replaced with "partition into" (because the "binding" work is more associated with events like binding to a receptor, not dissolving into a membrane).

Line 109 in the manuscript: "The MIE, "decrease, uncoupling of OXPHOS", is a lumped term representative of…": replace "uncoupling" by "coupling" in the MIE name.

Line 203 in the manuscript: insert "to" before "this"

Line 218: "… non-vertebrate models" – please specify, such as?

Line 233: "… relationships between uncoupling of OXPHOS and ATP synthesis …" However, what is critical to the final ATP pool is not only the ATP synthesis, but also ATP consumption processes – are there also models taking these into account?

Line 246: the authors might also consider the model developed for predicting fish growth based on cell proliferation, as described in Stadnicka-Michalak et al. 2015.

Page 3 of the AOP snapshot: at the top of the page in the tabular section on "Life Stage Applicability", the evidence for "Juvenile" is listed as "Not Specified." However, later on the same page, in the free-text section, juveniles are listed as known applicability domain, similarly as in several other pages in later sections (for example, page 10, evidence for Juvenile is given as "high"). Perhaps the first instance stating "unspecified" represents a typo and should be changed?

Page 10, in the section "Evidence for Perturbation by Stressor", in the first bullet point, insert "share" before "several", i.e. "These protonophores share several common…"

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Responses to reviewers - AOP 263 report

Dear Editor and Reviewers,

We appreciate the valuable comments and suggestions. Please find below our responses and revisions. The responses are shown in blue and the corresponding text revision is shown in green in the revised manuscript. We sincerely hope that the revised manuscript fulfills the requirements for publication in Environmental Toxicology & Chemistry.

Sincerely,
Dr. Song and Dr. Villeneuve
June 15, 2021
Oslo, Norway

❑ Responses to reviewers' comments

The reviewers commend the authors for the work carried out to prepare this submission. Uncoupling of oxidative phosphorylation (OXPHOS) is one of several important mechanisms that can lead to mitochondrial dysfunction and toxicity. The MIE "Decrease, Coupling of OXPHOS" is well characterized through multiple studies. The two KEs, "Decrease, Adenosine triphosphate pool" and "Decrease, Cell proliferation" are both at the cell/tissue level of biological organization. The AO "Decrease, Growth" can be assessed at various levels of biological organization, ranging from tissue to organism. Growth inhibition is an accepted regulatory endpoint, addressed, for example, by several OECD test guidelines (TG). In general, the reviewers agree with the AOP organization and description, as well as the assessments made by the authors with respect to the strength of evidence for individual KEs and KERs. However, several aspects, as listed below, might require further consideration and potential revision by the authors.

Major comments

1. First, not only the uncoupling of the OXPHOS, but also several other mechanisms could lead to dissipation of the proton-motive force (PMF). Therefore, it may be important to understand whether the observed effects on the PMF are a direct consequence of uncoupling or secondary to another mechanism. In the light of this, we invite the reviewers to consider capturing the "dissipation of the PMF" as a separate KE in this AOP. Inclusion of this event as a separate KE entity could allow intersecting/linking with any future AOPs that would describe mechanisms other than OXPHOS uncoupling that could also lead to PMF dissipation; otherwise, these might remain disconnected.

Response: We appreciate this good comment from the reviewers. We would still like to keep "decreased coupling of OXPHOS" as the MIE, without separating "dissipation of the PMF" as an independent MIE/KE, for the following reasons:

1) We consider a separation of "uncoupling of OXPHOS" and "dissipation of PMF" unnecessary, as we consider the former a lumped term and a consequence of the

latter. As explained in the manuscript (L109-113), the MIE of this AOP, decreased coupling of OXPHOS is a lumped term describing the **outcome** of several intermediate steps: binding of protons in the inter membrane space by an uncoupler, transportation of the protons across the inner mitochondrial membrane and dissipation of the PMF. To our understanding, we could either consider the term "uncoupling of OXPHOS" as a series of actions of a chemical (i.e., uncoupling action), or a final consequence of these processes (i.e., uncoupled oxidation and phosphorylation). It seems that the reviewers referred to the former (an uncoupler's actions leading to dissipation of PMF), rather than vice versa as we thought (i.e., chemical mediated dissipation of PMF leading to uncoupling of OXPHOS a consequence). We fully understand that stressors/mechanisms other than uncouplers/uncoupling actions can lead to dissipation of the PMF, such as abnormal cation (Ca^{2+}, K^{+}) influx into the mitochondria, proton slip within the proton pumps and impaired structural integrity of the mitochondria (Demine 2019). However, these mechanisms converge to dissipation of the PMF and eventually lead to uncoupled oxidation and phosphorylation due to loss of the driving force. What really matters for downstream biological processes, in most cases, is not dissipation of the PMF itself, but dissociation of oxidation with phosphorylation as the consequence of PMF dissipation. As an AOP is expected to capture the most critical events as the KEs, we consider "uncoupling of OXPHOS" as a more critical event than dissipation of the PMF determining the downstream effects.

2) As uncoupling of OXPHOS is normally not directly measurable, but indirectly indicated by the PMF (membrane potential) as one of the most frequently used approaches, separating PMF and uncoupling may not be very helpful in terms of quantification of the event.

3) "Uncoupling of OXPHOS" is a widely used term, representing a specific mode of action (MoA) of chemicals (i.e. uncouplers) familiar to the researchers, industry and regulators, potentially elevate the impact of this AOP, compared with having "dissipation of the PMF" as the MIE.

4) Compared with the choice of MIE/KE in other AOPs, for example, estrogen receptor agonism. There are multiple detailed processes upstream of the receptor activation, such as binding of chemical to the protein, different types of conformational change of the ligand binding pockets etc. But what really matters is the outcome, i.e., receptor activation. Therefore, the lumped term of ER activation (agonism) has been used as the MIE.

5) As AOPs are living document, this AOP is obviously not the final version and will be improved with the evolvement of knowledge and technology. We may still consider dissipation of the PMF as a separate MIE/KE if we really have the needs for differentiating these detailed processes.

Nevertheless, we would like to keep the final choice open for discussion, if the reviewers disagree with our arguments above. In this case, a meeting to discuss things through would be very helpful.

2. We notice that the "Background" section in the AOP snapshot is rather short. Perhaps this was intentional, while the "Introduction and background" section of the accompanying manuscript captures more information. However, we feel that also in the manuscript, the readers would certainly benefit from the addition of several important references that are currently missing. In particular, we refer the

authors to the following three publications: Ebert and Goss 2020: While this paper is purely mechanistic modeling to predict protonophoric uncoupling activity, it has an extensive list of references following the science of respiratory uncoupling. The authors should consider including some of these references in their overview; Schultz et al. 2002: This paper presents a comparison of pentachlorophenol (PCP) results with those elicited upon exposure to the model nonpolar narcotic 1-octanol, which revealed marked differences in both growth kinetics and the relative percentages of selected fatty acid methyl esters (FAMEs) in both pellicle and mitochondrial membranes; Hawliczek-Ignarski et al. 2017: This paper provides further evidence with regard to PCP's MoA. It is also a good example of how toxicogenomic data could be used to inform AOP development and which kind of testing data could be obtained with toxicogenomic approaches, so the 2 authors might also consider discussing this particular aspect as well, particularly in the section focused on the toxicity assays relevant for this AOP.

Response: Yes, it was intentional to have a longer background in the report than in the Wiki, as the report will target more general audience and the Wiki pages are supposed to present concise information related to this AOP. Additionally, "background" is an optional section on an AOP page, while it is part of the standard format of the more narrative AOP Report manuscript format. We appreciate the literature recommended by the reviewers and we have added these to support the AOP report.

Report, L43: added "(e.g., Schultz (2002)".

Report, L43: added "e.g., Sugiyama (2019))".

Report, L45-47: added "Predictive approaches such as quantitative structure-activity relationship (QSAR) (e.g., Dreier (2019a) and biophysical models (e.g., Ebert (2020)), and classification approaches such as toxicogenomics (e.g., Hawliczek-Ignarski (2017)) have been developed to identify new organic uncouplers."

3. Overall, we do not suggest that the reference list of the presented manuscript needs to approach 100 or even 50 references, but the final reference list should both reflect the history of the subject and identify the key publications along the way. For example, consider also the contributions by Hanstein 1976, McLaughlin and Dilger 1980, and Mitchell 1966. Lastly, we observed that in many cases the references cited in the AOP snapshot are not included in the manuscript. We feel that most of the omitted references would make for a useful addition to the manuscript as well, especially considering that the manuscript might reach a wider readership and therefore needs to be more extensively supported by references, compared to the entry in the AOP wiki.

Response: We thank the reviewers for the recommendations, and we have included these publications in the report. As instructed by ET&C, an AOP report is a "front matter" type of article, not a review. Therefore, the recommendation is to avoid citing all the detailed references used to support the AOP, but rather cite only those most pertinent to the narrative presented in the report with the understanding that interested readers and find more details and the associated references in the AOP-Wiki. We aim to incorporate a broader review of the history and state-of-the-art in this field to support the expansion of the present AOP into a broader AOP network.

Nevertheless, we have added the three references suggested by the reviewers to further support some of our statements in the report.
Report, L21: Added a citation "(Mitchell , 1966)"
Report, L26-27: Added a citation "(Hanstein 1976)".
Report, L41: Added a citation "(McLaughlin 1980)".

4. We also feel that the role and potential significance of the uncoupling proteins (UCPs) may be worth mentioning as well. UCPs are produced and regulated endogenously. Some UCPs play a role in heat generation, while others may play a role in modulating mitochondrial reactive oxygen species production (i.e., mild uncoupling, by decreasing the degree of reduction of ETC complexes, can decrease leakage of electrons to oxygen). See, for example, publication by Brand and Esteves 2005. We invite the authors to consider if the evidence on the effects of UCPs on some downstream events could further support the essentiality of those KEs?

Response: We completely agree with the reviewers that knowledge/data from the uncoupling proteins can be useful to support this AOP. However, we have not found any UCP studies that could provide direct evidence (i.e., essentiality, dose or temporal concordance) to support the proposed KEs or KERs in this specific AOP, albeit some UCP studies, such as Brand and Esteves 2005 can be useful for supporting other AOPs (e.g., the mtROS-centric ones) in the network.

5. Alternately, we see that down the road, the authors plan to connect uncoupling to mitochondrial ROS production. Inclusion of information on the connection of uncoupling to mtROS production may be more pertinent at that time. At this point, our understanding is that the relationship of mitochondrial uncoupling to mtROS production is that low levels of uncoupling decrease mtROS production, but high levels of uncoupling would eventually cause severe enough loss of mitochondrial homeostasis that mtROS increases. We suspect that this higher-"dose" effect may occur in the context of cytotoxicity, though, where mtROS production may be an effect rather than the proximate cause of mitochondrial dysfunction and cellular toxicity. However, a low-level decrease of mtROS levels could also be deleterious, especially in development, since mtROS signaling is important in developmental patterning and wound healing (Love et al. 2013; Timme-Laragy et al. 2018).

Response: We thank the reviewers for pointing out this important aspect. Since this AOP report specifically focuses on ATP-related effects, we have intentionally minimized the text related to other AOPs, such as the ROS pathways, but rather plan discuss them in detail in a subsequent AOP report on the broader AOP network that branches from the AOP described here.

6. The potential for uncoupling to trigger a compensatory increase in glycolysis should perhaps be mentioned as well. This mechanism has been observed/known for a long time (Weinbach 1957) and it may actually bypass or reduce an apparent decrease in ATP (see for example Bestman et al. 2015), yet still result in an overall decrease in energy availability and growth since glycolysis is less efficient compared to OXPHOS. This important point may also need a separate discussion/mentioning in the section on the overall evidence assessment for this AOP and its KEs and KERs, as well as in the section that discusses alternative tests for this AOP. This is because

there is also evidence that you can have compensatory upregulation of other energetic pathways, which will still come at a cost because this also requires energy. With this, you will not be observing an ATP decrease in vivo, although energy limitation would still be occurring, because of the overall less efficient use of available food resources. In vitro, if one would grow cells capable of glycolysis, uncouplers could appear much less toxic under these conditions than if the cells are forced to respire. See, for example, Marroquin et al. 2007.

Response: We completely agree that glycolysis as a compensatory mechanism needs to be mentioned. In fact, we have mentioned that glycolysis could be compensatory mechanism in L52-53. We also consider it more relevant to the uncertainties and quantitative understanding of the AOP. We have therefore added a few sentences in these sections to stress on the importance of glycolysis.

Report, L53: added a citation "Weinbach 1957".

Report, L231-236: added "Seventh, only a limited number of studies have considered glycolysis as a compensatory mechanism to uncoupling of OXPHOS. The ATP pool may show marginal changes due to activation of alternative ATP synthetic pathways such as glycolysis (Marroquin 2007). It should also be noted that the regulation of compensatory mechanisms may also require additional consumption of energy. Therefore, the observed change in ATP pool is expected to be influenced by multiple upstream biological processes."

Report, L241: added "such as glycolysis".

7. Page 4 of the AOP snapshot: Table for the Essentiality of the Key Events says that "There are currently no inconsistencies and uncertainties identified by the authors." However, the authors themselves have cited, for example, the case when ATP pool increases upon mild exposure to uncouplers. While the authors do offer an explanation for why this might be the case, should this not be considered a remaining uncertainty in this pathway, as long as the underlying mechanistic and quantitative relationships have not been characterized in more detail?

Response: We thank the reviewers for pointing out this, and we have revised the text in the Wiki.

Wiki, Essentiality-Inconsistencies & uncertainties: revised to "There is an uncertainty related to KE1446 that mild uncoupling of OXPHOS may also increase the ATP pool in some cases, possibly as a compensatory response. The underlying mechanism remains to be further elucidated."

8. Concerning the mentioned "AOP network," of which the AOP 263 is said to be a "core" part of: We understand that the "AOP Network" is part of AOP-Wiki, but this mention is perceived as a detractor from AOP 263. With regard to this network, it is at the moment not clear, how much of it is still purely theoretical and how much is already listed and well-described with accompanying evidence collected and presented in the AOP wiki. Overall, we feel that, since the manuscript also repeatedly refers to the AOP network in the wiki, then it should also – at least briefly – explain the status of other AOPs belonging to the overall "network" of the AOP 263. Furthermore, the authors need to explain why the presented AOP 263 forms "the core of a larger AOP network" (as compared to the other AOPs in the network – which specific quality or descriptor makes it "the core" of the whole

group?) We further observe that the network view contains connections to certain other forms of mitochondrial dysfunction (e.g., CIII and ATP synthase inhibition) but not others (e.g., CI, CII, CIV, redox cycling, Krebs cycle, etc.)—presumably, these will be added in the future? It would be helpful if the authors could comment on this as well.

Response: We agree with the suggestions and have added some text to briefly describing the network. We have also removed the "core" to avoid potential misunderstanding.

Report, L83-93: revised to "With the motivations and project support, a set of conceptual AOPs linking uncoupling of OXPHOS to growth inhibition were assembled and submitted to the AOPWiki (AOP 263-268). While the AOP presented herein (AOP 263) represents an important part of a broader network, it is understood that other intermediate key events may also contribute to growth inhibition. For example, generation of mitochondrial reactive oxygen species (mtROS) and subsequent oxidative stress due to abnormal redox reactions, impaired lipid metabolism due to loss of energy homeostasis, and programmed cell death due to oxidative damage can all occur as a result of OXPHOS uncoupling. The proposed AOP network therefore considers various consequences of mitochondrial uncoupling as key events, such as mtROS formation, DNA damage, protein oxidation, lipid peroxidation and cell death. Detailed description of the likely concurrent key events and their relationships to OXPHOS uncoupling and/or growth inhibition will be addressed elsewhere.

9. Looking at the list of the AOPs which include the MIE "Decrease, Coupling of OXPHOS" (on page 9 of the AOP snapshot), one cannot help but wonder whether all 6 (!) AOPs with practically identical names are truly necessary (i.e., all are called "Uncoupling of oxidative phosphorylation leading to growth inhibition", with numbers 1-6 included in the end). We feel that the authors should provide a more detailed explanation, both in the snapshot and in the manuscript, as to why they find this granular structure necessary and what are the benefits they expect to gain from the proposed formulation of this particular "network" consisting of closely related if not nearly identical pathways.

Response: We appreciate the reviewers for pointing this out. We have revised the full titles of the six AOPs but kept the numbers in the short titles (for them short enough).

AOPWiki, AOP 263-268 titles, revised to:

AOP263 - Uncoupling of oxidative phosphorylation leading to growth inhibition via decreased cell proliferation (Uncoupling of OXPHOS leading to growth inhibition 1)

AOP264 - Uncoupling of oxidative phosphorylation leading to growth inhibition via increased cell death (Uncoupling of OXPHOS leading to growth inhibition 2)

AOP265 - Uncoupling of oxidative phosphorylation leading to growth inhibition via decreased lipid storage (Uncoupling of OXPHOS leading to growth inhibition 3)

AOP266 - Uncoupling of oxidative phosphorylation leading to growth inhibition via oxidative DNA damage (Uncoupling of OXPHOS leading to growth inhibition 4)

AOP267 - Uncoupling of oxidative phosphorylation leading to growth inhibition via increased lipid peroxidation (Uncoupling of OXPHOS leading to growth inhibition 5)

AOP268 - Uncoupling of oxidative phosphorylation leading to growth inhibition via increased protein oxidation (Uncoupling of OXPHOS leading to growth inhibition 6)

Report, title: changed to "Uncoupling of oxidative phosphorylation leading to growth inhibition via decreased cell proliferation".
Report, Box 1, Formal AOP title: changed to "Uncoupling of oxidative phosphorylation leading to growth inhibition via decreased cell proliferation".

10. We point out that event 1771 is also supported by two other studies, i.e., Luz, Lagido, et al. 2016 and Luz, Godebo, et al. 2016, which showed that in vivo exposure of C. elegans to FCCP caused an increase in oxygen consumption coupled to a decrease in steady-state, in vivo ATP levels.

Response: We have included Luz, Godebo, et al. 2016 (measured both uncoupling and ATP) as empirical evidence to support the AOP, whereas excluded Luz, Lagido et al., 2016 (measured ATP) due to a lack of measurement for two adjacent KEs in the study.
Wiki, Relationship 2203 (KER1), Empirical evidence: added "Incidence concordance: Exposure of the nematode Caenorhabditis elegans to 50 µM Arsenite for 1h led to approximately 45% uncoupling of OXPHOS and 20% reduction in ATP (Luz 2016).
Wiki, Stressors: added "Arsenite" and evidence "High".
Wiki, Taxonomic Applicability: added "Caenorhabditis elegans" and evidence "Moderate".
Report, SI, Table S1: added incidence concordance from (Luz 2016).

11. With regard to the "overall assessment" for the KER3 of this AOP (event 1521): this event is supported by in vivo evidence from Bestman et al. 2015. This study has already been cited by the authors in support of the event 1821. However, the findings from this study are worth discussing with regard to event 1521 as well, because it reveals the unsurprising potential for cell- and tissue-specific effects to become larger when they are high-energy-use, potentially leading to teratogenesis in addition to growth inhibition (a mechanism that could perhaps form another AOP?). Therefore, the Bestman et al. 2015 reference should also be cited here as an in vivo example to make a point that there can be large tissue-specific effects and that not every cell type is equally susceptible. The authors should perhaps mention this as a placeholder, in order to ensure that the respective additional AOPs will at some point get constructed as well. These AOPs could also be seen as potential branching points to the AOP in question.

Response: We agree with this suggestion. But we consider this issue more as an uncertainty of the AOP. Therefore we have added a sentence to the uncertainty section.
Report, L210-211: added "There can also be large tissue-specific effects and that not every cell type is equally susceptible (e.g., Bestman (2015))."

12. Overall, we do understand that a single AOP cannot be expected to capture all related evidence. However, we also feel that it is quite important to find the right balance between the understandable desire of the authors to be succinct and describe only what's necessary, but at the same point to avoid a situation when the 'naïve' people who would come and read this description would walk away with a feeling that growth inhibition is the only effect that uncouplers might lead to, or that growth inhibition is only caused by uncoupling. Therefore, we feel that more granularity in the descriptions for some KEs, as well as some more details

provided when discussing the supporting evidence, might make for a valuable addition to the manuscript. Our main concern is that we do not want this pathway to be interpreted in isolation and therefore we feel that the potential additional mechanisms, as well as some conflicting evidence, should be properly mentioned and discussed as well. As a suggestion, the authors could consider adding a sentence to their Discussion section in the manuscript, which should explain that, while this particular AOP is focused on a specific, necessarily limited chain of events only, it is also recognized that there are additional outcomes possible. Some of these additional outcomes should then be listed as examples, without being exhaustive of course. The goal of this addition would be that a reader which is new to the field would at least become aware of the associated complexity. This could also open the door to some other AOPs to be developed. For example, the authors should discuss in more detail the link to teratogenesis as an outcome of growth disruptions manifested in certain organs. Indeed, the occurrence of both the malformed progeny as well as runts (smaller, often retarded siblings within one litter) are both caused by the effects related to developmental toxicity and dependent on cell proliferation capacity. Therefore, both can be seen as hazard endpoints that can be influenced by uncouplers. In the adult (mature) organisms, cell proliferation-related effects could also be particularly relevant for the tissues that maintain active proliferation status throughout life, e.g. gut which is always in a state of active turnover. In contrast, this certainly would not affect the brain, as you typically do not get more neurons. The latter point might again be true in mammals but much less so in fish, where neural tissue proliferation remains a life-long possibility. Some systemic effects (e.g., cardiac toxicity) also partially depend on cell proliferation. Overall, a better characterization of the AO at the organ level should capture some of this discussion. The authors should also add a sentence or two highlighting that there could be related outcomes other than organismal growth. Such discussion helping to relate the AO postulated in this AOP to some of the more traditional in vivo endpoints has the potential to further improve the presented AOP and its usefulness in the context of risk assessment.

Response: We appreciate this excellent point raised by the reviewers. However, rather than place this in the discussion, we feel it is important to emphasize up front in the background and introduction section to avoid an impression that growth inhibition is the only relevant outcome as the reader proceeds through the report. We have therefore added two sentences immediately following the introduction of the putative AOP network.

Report, L93-98: added "It should also be noted that growth inhibition is likely not the only adverse outcome following uncoupling of OXPHOS. Other types of uncoupler mediated adverse effects, such as teratogenesis, reduced fertility, neurodegeneration and cardiac diseases may also be considered when assembling information into a larger AOP network for mitochondrial dysfunction. While the present report focuses growth inhibition, that is just one of several potential adverse outcomes that may be linked to uncoupling of OXPHOS."

13. Further, on page 3 of the AOP snapshot, in the section on the life stage applicability domain, the authors state that "Classical uncouplers such as 2,4-DNP have been reported to cause weight loss in adult humans […] suggesting that

adults are partially in the applicability domain of this AOP." This statement can and probably should be further strengthened. In fact, 2,4-DNP was sold legally for this purpose (i.e., weight loss), until its legal sale was banned because some people took too much of it and died as a result. This chemical, however, is still available online, and still killing people, unfortunately, see e.g. the report by Baker and Baker 2020. Therefore, human adults are indeed affected and susceptible to the effects of OXPHOS uncouplers.

Response: We thank the reviewers for pointing out this and have revised the Wiki page accordingly.

Wiki, Life stage applicability domain: revised to "Classical uncouplers such as 2,4-DNP have been reported to cause weight loss in adult humans (Grundlingh 2011). In fact, 2,4-DNP was sold for weight loss until its legal sale was banned over toxicity and abuse concerns (Baker 2020). These suggest that adults are in the applicability domain of this AOP."

Wiki, Life Stage Applicability: added "Adult" and evidence "High".

14. The susceptibility of adult humans to mitochondrial uncoupling is further supported by what appears to be the first report of a (genetic) mitochondrial disease in people, namely the Luft Disease (Luft et al. 1962). This disease is thought to be caused by mitochondrial uncoupling (unfortunately, the specific gene(s) responsible for this mechanism remain unidentified) and is characterized by hyperthermia, perspiration, and enormous appetite despite low weight. The patient was underweight as a child, despite increased appetite. Again, this example further supports the idea that the uncoupler effects have high human health relevance. Likely beyond the scope here, but perhaps worth keeping in mind as well: there is currently evidence of mitochondrial uncoupling leading to either increased or decreased neurodegeneration—perhaps related to the non-monotonic effects on mtROS. This could also be another AOP to be developed later.

Response: We appreciate this good comment. We will certainly include the mtROS pathways as well as neurogenerative AOs in a larger network related to mitochondrial uncoupling in the subsequent work.

15. Overall, we strongly emphasize that for this AOP and the associated effects, the environmental (ecotoxicological) and human health aspects should not be discussed in isolation. Currently, we observe a certain tendency of this AOP to lean more towards discussing the ecotoxicological aspects and applications, while the potential human health effects have been discussed rather cursorily and without going into much detail. We feel that there should be more discussion related not only to environmental health concerns but also to human health concerns, in order to better outline how these findings are specifically related to human health. We do understand that this AOP might have more of an ecotox flavor based on the authors' main expertise, but we do encourage them to expand it according to the directions suggested above.

Response: We appreciate the comment. In fact, we did not intend to differentiate the ecological and human health for this AOP. On the contrary, we would like this AOP to cover both aspects. AOP 263 has therefore been highly general to cover as many

eukaryotes as possible. As shown in the concordance table (SI), we have used evidence from both human (cells and cancer studies) and ecological species to support this AOP. We greatly appreciate the authors suggestions for additional literature to support the present AOP and have incorporated those additional references where appropriate.

16. On the other hand, with regard to ecotoxicological applications of this AOP, we were also somewhat surprised to observe that, while the authors do talk about growth on the tissue, organ and organismal level, they have not outlined any potential connection to population-level outcomes. At the same time, this AOP does place a lot of emphasis on its ecotoxicological relevance, as we have just discussed above. Therefore, we consider that it would be valuable if authors also compiled the evidence available with regard to potential population-level effects as well.

Response: We also agree that population effect can be a potential downstream AO of this AOP. Nevertheless, we do not intend to include population decline as another AO at the moment, as that particular linkage has been long accepted within the field of ecotoxicology to the point where it is accepted as canonical knowledge. Development of such a KER, while potentially valuable for many AOPs, is likely best accomplished by investigators with extensive experience in population modeling and management (e.g., fish and wildlife management specialists).

17. Further, with regard to environmental relevance, the authors should please elaborate on the significance of a lower acute-to-chronic ratio (ACR) for uncouplers (mentioned at line 255 in the manuscript). An important point to make here could be to explain, what the potential consequences of that could be.

Response: The cited ACRs for growth and uncoupling are not directly comparable, as the former was calculated for all types of chemicals and one endpoint, whereas the latter was calculated for uncouplers and multiple endpoints. The purpose of citing these values was to demonstrate that environmentally realistic levels of uncouplers could trigger the AOP (as stated in L277-278). We have nevertheless revised the text to further clarify the statements.

Report, L275: added " for all documented chemicals affecting growth".

Report, L276: added "leading to various types of adverse effects in different organisms".

18. With regard to the sex applicability domain of this AOP, we encourage the authors to consider including a study looking at PCP-caused decreased growth in rats (Schwetz et al. 1978), as there are also some sex-specific effects described in this paper.

Response: We have included this publication in the report.

Report, L278-279: added "The response patterns of the KEs, however, can vary dramatically between males and females (e.g., Schwetz (1978))."

19. The statement on Page 3 of the AOP snapshot, "The chemical applicability domain of the AOP mainly includes weak acids, such as …." is accurate, but perhaps it would also be helpful to explain why this is the case. That is, describe that uncouplers typically have properties as both weak acids and hydrophobic

substances. As weak acids, they are capable of gaining and losing an electron. As hydrophobic substances, they are capable of distributing a negative charge over a number of atoms (often by π-orbitals which delocalize a proton's charge when it attaches to the molecule), so that they can diffuse back and forth across the IMM in either the charged or uncharged state, thus moving protons back across the concentration gradient generated by the ETC. A more detailed discussion of these mechanisms could be useful for any future analyses by scientists who might be interested to apply physicochemical property analysis to discovery of uncouplers.

Response: We agree with this suggestion and have added text in the Wiki page accordingly.

Wiki, Domain of applicability: added "Uncouplers typically have properties as both weak acids and hydrophobic substances. As weak acids, they are capable of gaining and losing an electron. As hydrophobic substances, they are capable of distributing a negative charge over a number of atoms (often by π-orbitals which delocalize a proton's charge when it attaches to the molecule), so that they can diffuse back and forth across the inner mitochondrial membrane in either the charged or uncharged state, thus moving protons back across the concentration gradient generated by the electron transport chain."

20. We also observe that the chemical applicability domain of this AOP, which appears to be mainly focused on weak acids, might be unnecessarily narrow. It is not completely clear to us if hydrophobic ion or SH-reactive types of uncouplers have been considered/included as well. It would be helpful if the authors could clarify this point.

Response: We have added a sentence to state the inclusion of these types of uncouplers in the applicability domain of the AOP.

Wiki, Domain of applicability: added "Other types of uncouplers that are SH-reactive chemicals or hydrophobic ions may also be in the applicability domain of this AOP."

21. We further note that historically (e.g., in the cases of AOPs on skin sensitization or AOPs for estrogen-mimicking substances), AOPs have always included some discussion of applicability domains. However, one also needs experimental data on classic uncouplers within themselves to suggest an applicability domain. For example, 2,4-dinitro-, pentachloro- and 3,5-dichloro-phenol suggest the phenolic weak acid domain. But not all nitro/chlorophenols are uncouplers of sufficient strength to decrease growth before death occurs. In our view, one of the seminal functions of an AOP could be guiding direct testing to define the boundaries of its applicability domains.

Response: We agree with this comment and have added one sentence in the report to present this thought.

Report, L291-292: added "One of the seminal functions of this AOP could be guiding direct testing to define the boundaries of its applicability domains."

22. With regard to the section on alternative assays: It is noted that three out of four KEs in this AOP can be measured using high-throughput in vitro assays. We were wondering if data from these assays could also be used as empirical support for

the key event relationships? We suggest that, for assays that capture multiple key events, this information could be added to the concordance table, i.e. Table S1 in the supplementary material, as these would constitute useful additional lines of evidence for the key event relationships. For further information on the multiplexed assays, see Shah et al. 2016.

Response: Yes, we strongly agree with the reviewers that the ToxCast/Tox21 data can be quite useful for supporting empirical concordance of this AOP. We have performed some data mining searching for more evidence. Unfortunately, we have not found a good dataset reporting simultaneous measurement of at least two adjacent KEs in this AOP (not even from Shah et al., 2016). We consider the current concordance table being sufficient for the report. As AOPs are living documents, the current concordance table is absolutely not the final version. We will continue to add evidence to the concordance table with the progression of this research field.

23. We also observe that a more detailed description of assays that could be used and would be important for the endpoints associated with the MIE and KEs in the outlined AOP is very critical, because this will go through the OECD. For applications there, it is not enough to just have a pathway, but you also need to have the assays with which it can be measured. Consequently, an AOP could be stuck at OECD if there are no good ways to measure an important KE. Therefore, better outlining these assays would be a critical point to move forward with this AOP.

Response: We appreciate this comment and agreed that the AOP itself should be a testing strategy or description toolbox. In fact, we have connected this AOP to established ToxCast/Tox21 assays (for MIE, KE1 and KE2) and OECD test guidelines (for AO). Therefore, we did not want to repeat the assay descriptions in this report or in the AOP-Wiki, but rather redirect the readers/users to the ToxCast/Tox21 and OECD protocols by citing their IDs.

Report, Box2-MIE: added "Standardized ToxCast high-throughput screening bioassays, such as "APR_HepG2_MitoMembPot", "APR_Hepat_MitoFxnI", and "APR_Mitochondrial_membrane_potential", and the Tox21 high-throughput screening assay "tox21-mitotox-p1" can be used to measure this MIE."

Report, Box2-KE1: added "Standardized ToxCast high-throughput screening bioassays, such as "NCCT_HEK293T_CellTiterGLO" and "NIS_HEK293T_CTG_Cytotoxicity" can be used to measure this KE.

Report, Box2-KE2: added "Standardized ToxCast high-throughput screening bioassays such as "BSK_3C_Proliferation", "BSK_CASM3C_Proliferation" and "BSK_SAg_Proliferation" can be used to measure this KE."

Report, Box2-AO: added "Standardized OECD test guidelines (TGs), such as TG206, 208, 201, 210, 211, 212, ,215, 221, 228 and 241 can be used to measure this AO in different species."

24. AOP snapshot, page 2, the section on stressors: "moderate" evidence is given for pentachlorophenol while "high" is mentioned for all other listed chemicals. We were not able to locate a clear explanation for why the evidence for PCP is only moderate. In this regard, we also invite the authors to consider PCP-related evidence from studies by Schultz et al. 2002 and Hawliczek-Ignarski et al. 2017.

Response: We agree with the reviewer's comment and have revised it accordingly.
Wiki, Stressors: revised evidence level to "high" for PCP.

25. Further with regard to regulatory significance and potential applications of this AOP: We observe that the AO "Decrease, Growth" refers to growth inhibition, which is accepted as a regulatory endpoint in many countries (though not all) and has been addressed by several OECD test guidelines (TG). In a regulatory context, effects on growth can be measured with parameters such as length, wet or dry weight, or as a rate over time (as is common in algae). The authors do list some of these TGs in the section "Regulatory significance of the AO." This section could be further expanded to also include guidelines for chronic toxicity testing in fish (TG 210) and birds (TG 206), thereby improving the applicability of this AOP as a framework for animal alternative approaches. In addition, the authors may consider adding a short discussion of the main differences in legislative mandates that some countries have with regard to growth as a regulatory endpoint. Overall, we feel that the discussion in this section should be expanded to explain how this AOP relates to the real world in terms of regulatory practice. For example, the authors could provide concrete examples linking different organisms to the listed TGs, as this would allow different regulatory bodies from across the world to better relate to this particular AOP.

Response: We have added the two TGs in the list and provided more details about the taxonomic groups that are covered by these guidelines. The text in the "considerations for potential applications of the AOP" section in the AOP-Wiki was also revised to note that growth is a recognized endpoint of concern in OECD member countries. It is beyond the scope of our AOP and expertise to provide a comprehensive review of the recognition of growth as an endpoint in all countries or in human health contexts, but we would certainly invite the reviewers or others to suggest additional details that would be relevant to add to this section.

26. While the 'consideration for potential application of the AOP' is optional in the AOP-Wiki, we deem it highly critical to publication in ET&C. We suggest that each of the presented considerations should be discussed in more detail in the manuscript. That is, not just listing with one sentence, but elaborating and presenting additional evidence and further considerations, as well as concrete examples or potential case studies for each point, where available. With regard to the presented considerations themselves, we agree with most of them. One exception, however, is the fourth consideration, stating that the AOP is "highly generalized and has wide biological and stressor applicability domains, making it a central hub for many other AOPs." We understand that this consideration may stem from the assumptions and expectations associated with the previously mentioned "AOP network." However, we feel that this is rather speculative, as no specific proofs have been provided so far and we are not completely convinced of the utility or applications of this particular network (see also above for additional considerations regarding the "network" aspect). We also suggest that the authors try to better illustrate the connections and interdependencies between the points raised. For example, linking considerations 3 and 5 should be emphasized, as this seems to be the classic way that the AOP provides the mechanistic/mode of action

plausibility/probability needed to identify the most endpoint relevant and key event-related test systems, which, when used, could help define the boundaries of 2D structure applicability domains and establish structure alerts for predicting potency by read-across or QSAR.

Response: We understand the view of the reviewers that it would be helpful to cite specific examples of the application of this AOP in the various contexts described. However, this AOP has just been developed and is currently under technical review. To our knowledge, no one has explicitly applied it in any of the context. The section is indeed speculative, as we are speculating about the potential applications of this AOP as we the developers conceive them. We do profess to know or have evidence for how this AOP may or may not be used by others in the future. We suggest several concepts here as a guide, but acknowledge this is by no means comprehensive. To recognize these points, we have modified the text of the "Considerations for potential applications of the AOP" section in the Wiki. We also invite users of this AOP to share their applications via the Discussion page in the AOP-Wiki so that practical examples may be added in the future. L285-297 provide a more detailed elaboration of the listing of potential applications summarized on L298-300. At present we are unaware of more specific case studies to illustrate applications of the present AOP. The potential utility for defining the boundaries of a 2D structure applicability domain to establish structure alerts for predicting potency by read-across or QSAR is more a function of the availability of a high throughput assay to detect uncouplers than it is the subsequent events and relationships in the AOP. Thus, we do not feel that particular example is distinct from the application that is already described on lines 285-288. We do concur with the reviewers that the potential application of the AOP as a hub for development of additional AOPs in a related AOP network is probably not important here. Mention of this point has been removed from both the AOP Report and the AOP-Wiki.

Wiki, Considerations for potential applications of the AOP: added ""This is a range of potential applications that were conceived during the development of the present AOP. However, it is neither an exhaustive list of potential applications, nor can explicit examples of these applications in practice be cited at this time."

Report, L300: deleted "4) serve as a central hub for other relevant AOPs."

27. One final consideration that came to our mind: can it be identified, which KE (or an MIE) represents the rate-limiting step in this AOP? This thinking was triggered by the estrogen-mimic AOP where ER-binding is the rate-determining step and fish liver vitellogenesis assay confirms this. The male-to-female gonadal conversion, feminization of male fish, and reproductive impairment are all downstream events that added weights-of-evidence to that AOP, but data for these events are not needed to make a regulatory decision. However, perhaps these considerations are going a step too far?

Response: We appreciate this excellent point from the reviewers. Our supporting project (RiskAOP, https://www.niva.no/en/projectweb/riskaop) for this AOP aims to answer the question and develop quantitative AOP models.

28. Thank you for providing the Tox21 data in the supplementary table S2. The assay documentation indicates this assay measures the mitochondrial membrane potential, and ATP content is used to measure cell viability in the assay (Attene-Ramos et al. 2015). If possible, it would be useful to include the cell viability data to discern specific effects on the mitochondrial membrane potential from general cytotoxicity. Providing both measures would give a clearer context for interpreting these data. Additionally, it is important to note this assay does not measure uncoupling directly, but rather, quantifies changes in the mitochondrial membrane potential as a potential consequence of uncoupling. Indeed, this information has been used to prioritize substances for additional mechanistic studies to identify uncouplers (Xia et al. 2018). It may also be important to note other high-throughput screening assays, such as respirometric screening assays, that can be used to identify specific mechanisms of action, including uncoupling (Hallinger et al. 2020).

Response: We thank the reviewers for this excellent point. We have indeed included the viability data in column 3 (SAMPLE_DATA_TYPE) in table S2. “Values of 'viability' for SAMPLE_DATA_TYPE indicate that the data represents the viability of the cells when exposed to the compound”. We also agree that respirometric screening of mitochondrial uncoupling is also a good approach to measure uncoupling of OXPHOS and we have mentioned it (Seahorse XF Analyzer) in Box2-MIE in the report. In out opinion, as high-throughput respirometers such as Seahorse analyzers can be a significant investment for a normal laboratory, measurement of membrane potential using fluorescent probes might still be a cost-effective choice, albeit relatively (Luz 2016)less accurate.

Minor comments

29. In the sentence “A number of chemicals can bind to the inner mitochondrial membrane” (in the Background section), “bind to” should be replaced with “partition into” (because the “binding” work is more associated with events like binding to a receptor, not dissolving into a membrane).

Response: Agree.

Report, L97: revised to “partitioning of protonophores (uncouplers) into the inner mitochondrial membrane”.

30. Line 109 in the manuscript: “The MIE, “decrease, uncoupling of OXPHOS”, is a lumped term representative of…”: replace “uncoupling” by “coupling” in the MIE name.

Response: Agree.

Report, L109: Revised accordingly.

31. Line 203 in the manuscript: insert “to” before “this”

Response: Agree.

Report, L203: Revised accordingly.

32. Line 218: “… non-vertebrate models” – please specify, such as?

Response: Agree.

Report, L218-219: added "such as insects, crustaceans, nematodes and mollusks."

33. Line 233: "… relationships between uncoupling of OXPHOS and ATP synthesis …" However, what is critical to the final ATP pool is not only the ATP synthesis, but also ATP consumption processes – are there also models taking these into account?

Response: We thank the reviewer for pointing this out. Yes, there are some studies investigating the relationships between ATP synthesis and consumption, but not necessarily under the OXPHOS uncoupling situation. We have cited a representative study in the report.

Report, L237-238: added "There are also case-specific studies reporting the quantitative relationship between ATP synthesis and ATP consumption in vertebrates (e.g., Matsuda (2020))".

34. Line 246: the authors might also consider the model developed for predicting fish growth based on cell proliferation, as described in Stadnicka-Michalak et al. 2015.

Response: Agree.

Report, L246: Cited Stadnicka-Michalak et al., 2015.

35. Page 3 of the AOP snapshot: at the top of the page in the tabular section on "Life Stage Applicability", the evidence for "Juvenile" is listed as "Not Specified." However, later on the same page, in the free-text section, juveniles are listed as known applicability domain, similarly as in several other pages in later sections (for example, page 10, evidence for Juvenile is given as "high"). Perhaps the first instance stating "unspecified" represents a typo and should be changed?

Response: Agree.

Wiki page, Life stage applicability: Changed evidence level to "High" for juveniles.

36. Page 10, in the section "Evidence for Perturbation by Stressor", in the first bullet point, insert "share" before "several", i.e. "These protonophores share several common…"

Response: Agree.

Wiki page, Evidence for Perturbation by Stressor: Revised accordingly.

Other revisions

-**Report, L73**, changed "of" to "for"

-**Report, L221**, changed "similar to" to "like"

References

Demine S, Renard P, Arnould T. 2019. Mitochondrial Uncoupling: A Key Controller of Biological Processes in Physiology and Diseases. *Cells* 8:795.

Luz AT, Godebo TR, Bhatt DP, Ilkayeva OR, Maurer LL, Hirschey MD, Meyer JN. 2016. Arsenite Uncouples Mitochondrial Respiration and Induces a Warburg-Like Effect in Caenorhabditis elegans. *Toxicol Sci* 154:195-195. DOI: 10.1093/toxsci/kfw185.

Second review

This review responds to revisions undertaken by the authors of the AOP263 "Uncoupling of oxidative phosphorylation leading to growth inhibition" and the accompanying ET&C manuscript in response to the initial review of the original submission that we have provided in May 2021. The four reviewers David Dreier, Ksenia Groh, Joel Meyer and Terry Schultz have jointly discussed, prepared, and approved the final review text below.

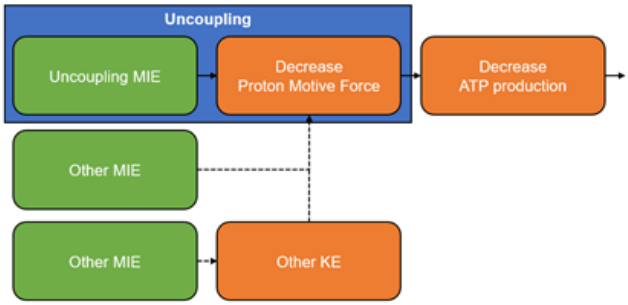
The reviewers feel that the authors have provided convincing explanations and/or revisions in response to most of the points raised in the initial review. We have only one major concern remaining, along with two other minor comments and a few suggestions for minor edits that we would like to share below.

Our major remaining concern is about the authors' argument that uncoupling should remain grouped ("lumped together") with decrease of proton motive force (PMF). We still think that dissipation of PMF should be included as a separate KE, as we recommended previously. We emphasize that decrease of PMF should be included as a KE, and not as an MIE—we cannot quite follow the authors' idea that loss of PMF leads to uncoupling, as we think it is actually the other way around, at least usually. With the latter point we specifically refer to the authors' statement "What really matters for downstream biological processes, in most cases, is not dissipation of the PMF itself, but dissociation of oxidation with phosphorylation *as the consequence* of PMF dissipation" (emphasis ours). We actually do not think there is clear evidence available to say that this is true.

In fact, it is the PMF itself (and not the downstream phosphorylation of ADP) that is critical for many biological processes (e.g., import of mitochondrial proteins; mitophagy; ion exchange; etc). In addition, a very strong evidence for the importance of maintaining membrane potential per se is the evolution of the possibility of cells burning ATP to run ATP synthase in reverse in order to maintain membrane potential.

We believe that some of this confusion may have derived from somewhat different literature definitions of the term "uncoupling"—e.g., in the Arnould review that the authors cite, it is defined as "a dissociation between mitochondrial membrane potential generation and its use for mitochondria-dependent ATP synthesis". However, more commonly, "uncoupling" is defined as uncoupling oxygen consumption from ATP production—which may or may not actually result in PMF loss. We were also wondering if another source of confusion regarding the sequence of events could stem from AOP311 (which is also being developed by one of the authors). Though that AOP is dealing with a slightly different mechanisms (and it is currently not open for review), we politely observe that there is certainly an opportunity to harmonize e.g. the names of some KEs there and we hope that our review for AOP263 could set a precedent for the required clarity of definitions.

Based on the considerations above, we maintain that uncoupling and PMF loss should not be "lumped" into one event. Our specific suggestion, depicted in the diagram below, would be to split the broader process of uncoupling (in blue) into a specific MIE and KE, with uncoupling (green MIE) leading to a decrease in the PMF (orange KE).



The authors said that they want to group this into a broader process, because "uncoupling of OXPHOS is normally not directly measurable." However, we believe that having PMF decrease listed as a separate KE would not preclude using the respective measurements to inform this particular AOP. Hence, we still think it would be valuable to have the PMF dissipation as a separate KE, as this would allow accommodating other upstream MIEs/KEs (uncoupling or otherwise) and provide an opportunity for future research instead of artificially limiting the possibilities to link additional MIEs/KEs to this particular AOP. Again, we do understand the desire to group these events for the sake of simplicity, but hope nonetheless that the authors will also see this is as an opportunity to "future-proof" their AOP.

Provided the authors agree to follow the suggestions above, sentence on page 3, lines 109-110 of the revised manuscript would also need to be revised. Namely, from "partitioning of protonophores (uncouplers) into the inner mitochondrial membrane is known to uncouple OXPHOS by dissipating proton motive force, leading to reduced ATP synthesis" to "partitioning of protonophores (uncouplers) into the inner mitochondrial membrane is known to uncouple OXPHOS, leading to dissipation of proton motive force and subsequent reduction in ATP synthesis."

Lastly to the above-discussed topic, we invite the authors to consider whether it is justified to postulate the "coupling of OXPHOS, decrease" as an MIE, although it is in fact preceded by another—truly initiating—event, which authors have also identified themselves, namely the "partitioning of protonophores (uncouplers) into the inner mitochondrial membrane"? Categorizing the uncoupling of OXPHOS as a KE instead of an MIE would align with the fact that it can be caused by several different mechanisms apart from the protonophores-dependent one. Hence, this could essentially create "space" for other MIEs to feed into the same KE "decreased coupling of OXPHOS".

Next, with regard to the authors' response to our comment number 16 in the initial review, we must say that we still do not completely understand the reasons for the authors' decision to not include population decline as a second AO in their AOP, despite the fact that, as they state themselves, "that particular linkage has been long accepted within the field of ecotoxicology to the point where it is accepted as canonical knowledge." If this is "canonical knowledge" indeed, then why wouldn't the authors acknowledge this and add the second AO? In other words, we were wondering if there are no other AOPs in the AOPwiki currently that have already sufficiently characterized the link from the AO "growth, decrease" to the AO "population, decline"? And if yes, could the authors "reuse" this particular relationship in their own AOP? After all, the possibility to "reuse" the already-existing KEs and KERs is one of the main advantages offered by the AOPwiki, hence we feel that the authors could have made a conscious effort to promote this practice.

Lastly, with regard to the authors' response to our comment number 15 in the initial review, we would like to share that we still feel that the descriptions accompanying this AOP continue to have a strong environmental bias, while human health applications are less visible. This is okay in the end, as this simply reflects the authors' main expertise. We, however, suggest that the authors consider adding a clear upfront statement acknowledging this and explicitly postulating that this AOP does have both the environmental and human health application.

Minor edits suggested:

Page 3, line 102: insert "on" before "growth" to have "focuses on growth inhibition"

Page 6, line 221: delete "that" before "not every"; should become "There can also be large tissue-specific effects and not every cell type is equally susceptible […]"

Responses to reviewers #2 - AOP 263 report

Dear Editor and Reviewers,

We appreciate your further comments and suggestions. We have some preliminary responses prior to making large changes. The report and AOP-Wiki will be revised accordingly after our final discussion.

Sincerely,
Dr. Song and Dr. Villeneuve
July 19, 2021

❑ Responses to reviewers' comments

1. **Separating "Uncoupling KE" (Event 1446) into two KEs:** Our major remaining concern is about the authors' argument that uncoupling should remain grouped ("lumped together") with decrease of proton motive force (PMF). We still think that dissipation of PMF should be included as a separate KE, as we recommended previously. We emphasize that decrease of PMF should be included as a KE, and not as an MIE—we cannot quite follow the authors' idea that loss of PMF leads to uncoupling, as we think it is actually the other way around, at least usually. With the latter point we specifically refer to the authors' statement "What really matters for downstream biological processes, in most cases, is not dissipation of the PMF itself, but dissociation of oxidation with phosphorylation as the consequence of PMF dissipation" (emphasis ours). We actually do not think there is clear evidence available to say that this is true. In fact, it is the PMF itself (and not the downstream phosphorylation of ADP) that is critical for many biological processes (e.g., import of mitochondrial proteins; mitophagy; ion exchange; etc). In addition, a very strong evidence for the importance of maintaining membrane potential per se is the evolution of the possibility of cells burning ATP to run ATP synthase in reverse in order to maintain membrane potential. We believe that some of this confusion may have derived from somewhat different literature definitions of the term "uncoupling"—e.g., in the Arnould review that the authors cite, it is defined as "a dissociation between mitochondrial membrane potential generation and its use for mitochondria-dependent ATP synthesis". However, more commonly, "uncoupling" is defined as uncoupling oxygen consumption from ATP production—which may or may not actually result in PMF loss. We were also wondering if another source of confusion regarding the sequence of events could stem from AOP311 (which is also being developed by one of the authors). Though that AOP is dealing with a slightly different mechanisms (and it is currently not open for review), we politely observe that there is certainly an opportunity to harmonize e.g. the names of some KEs there and we hope that our review for AOP263 could set a precedent for the required clarity of definitions. Based on the considerations above, we maintain that uncoupling and PMF loss should not be "lumped" into one event. Our specific suggestion, depicted in the diagram below, would be to split the broader process of uncoupling (in blue) into a specific MIE and KE, with uncoupling (green MIE) leading to a decrease in the PMF (orange KE). The authors said that they want to group this into a broader process, because "uncoupling of OXPHOS is normally not directly measurable." However, we believe that having PMF decrease listed as a separate KE would not preclude using the respective measurements to inform this particular AOP. Hence, we still think it would be valuable to have the PMF dissipation as a

separate KE, as this would allow accommodating other upstream MIEs/KEs (uncoupling or otherwise) and provide an opportunity for future research instead of artificially limiting the possibilities to link additional MIEs/KEs to this particular AOP. Again, we do understand the desire to group these events for the sake of simplicity, but hope nonetheless that the authors will also see this is as an opportunity to "future-proof" their AOP.

Response: We appreciate the reviewers' perspective regarding the potential to split our current generalized KE of "Coupling of OXPHOS, decrease" into two KEs, "Uncoupling, increase" and "Decreased proton motive force". Scientifically, we completely agree with the reviewers regarding the sequence of events in this pathway, i.e., partitioning of chemical into the inner mitochondrial membrane→transport protons across the membrane (uncoupling action)→dissipation of proton motive force→reduce phosphorylation of ADP into ATP (ATP synthesis). The key point that warranted further discussion was whether "dissipation of PMF" was considered part of the "general uncoupling mechanism of action", or a separate downstream event of the actual "uncoupling action" of a chemical. We considered that the former term had been generally accepted and a natural outcome of this was "Decrease, coupling of OXPHOS" – the MIE we initially proposed. We also concur that there would be several advantages relative to the ability to link a more specific MIE to chemical categories and for potential "future-proofing" in an AOP network context by splitting the current MIE into two events. However, we note with respect to "future proofing", AOPs 311 and 387 are both under development by our group. They will be harmonized to whichever decision is made regarding AOP 263.

That said, we have **one major concern** about splitting the MIE. That relates to **measurability**. We are not aware of any way to experimentally measure (quantify) or to computationally predict the "uncoupling action" (at least in a practical sense that would be feasible and accessible in many labs) independent of the dissipation of proton motive force. We are aware of using other downstream events, such as oxygen consumption at different respiration states, P/O ratio etc. to indirectly indicate uncoupling of OXPHOS in general. That said, given the reviewer's expertise in this area of biology, we concede that there may be approaches we are not familiar with. If a reasonable measurement method, independent of dissipation of proton motive force were identified, we would find it reasonable to separate "uncoupling" from "decreased PMF". Likewise, we note in the "chemical applicability domain" section of our many of the physical-chemical properties of probable uncouplers are well defined and there are in silico approaches to identifying potential uncouplers. Thus, in the context of an in silico approach to chemical screening as relevant methodology for identifying compounds likely to activate the KE of "Uncoupling, increase", the in silico prediction capabilities may warrant separation of the KEs, particularly if subsequent measurement of decreased PMF would be regarded as a way to lend strength to that prediction (i.e., in a tiered screening/testing approach). Our minor concern about splitting the MIE is the availability of empirical evidence (essentiality call, temporal, dose and incidence concordance) to support the relationship between "uncoupling" and "dissipation of PMF" (as normally the latter was measured to indicate the former), despite its high biological plausibility.

If indeed there is no real way to independently measure uncoupling independent from the near immediate (or nearly by definition result) of decreased PMF, separating the KEs – while it may be meaningful in terms of an accurate description of the biology, may be completely irrelevant from an application perspective. Thus, we would favor maintaining the single KE, and could address the two underlying mechanistic steps by adding additional "**Key Event Component**" terms (Eves et al., 2017) that would capture both the uncoupling process and the consequent reduced PMF. The original Event Component publication proposed that: "Multiple Event Components - KEs as currently defined based on the existing guidance tend to capture a broader portion of the biological system than can be defined using a single

biological process or object. As a result, to fully describe the KE using explicit biological entities, multiple Event Components may be needed with at least one required. This provides more flexibility in describing the KE at early stages of AOP development. Over time, it may be determined that some KEs should be split while others should be merged. Having formal descriptions of Event Components should assist with this process." The Key Event Component function has already been implemented in the AOPWiki (e.g., https://aopwiki.org/events/309, https://aopwiki.org/events/381). In this case, we propose to keep the lumped MIE term of "decreased coupling of OXPHOS", but differentiating "diffusion across the IMM and transport protons out (uncoupling action)" and "dissipation of PMF" as two event components associated with this MIE. These event components can be easily connected to other AOPs and further separated as independent KEs when appropriate.

2. **Minor revision to the text if Event 1446 is separated into Two Events:** Provided the authors agree to follow the suggestions above, sentence on page 3, lines 109-110 of the revised manuscript would also need to be revised. Namely, from "partitioning of protonophores (uncouplers) into the inner mitochondrial membrane is known to uncouple OXPHOS by dissipating proton motive force, leading to reduced ATP synthesis" to "partitioning of protonophores (uncouplers) into the inner mitochondrial membrane is known to uncouple OXPHOS, leading to dissipation of proton motive force and subsequent reduction in ATP synthesis."

Response: Agree and we will revise this sentence accordingly.

3. **Proposed event "Partitioning of the protonophore to the inner mitochondrial membrane":** Lastly to the above-discussed topic, we invite the authors to consider whether it is justified to postulate the "coupling of OXPHOS, decrease" as an MIE, although it is in fact preceded by another—truly initiating—event, which authors have also identified themselves, namely the "partitioning of protonophores (uncouplers) into the inner mitochondrial membrane"? Categorizing the uncoupling of OXPHOS as a KE instead of an MIE would align with the fact that it can be caused by several different mechanisms apart from the protonophores-dependent one. Hence, this could essentially create "space" for other MIEs to feed into the same KE "decreased coupling of OXPHOS".

Response: We do not feel that "partitioning of protonophores (uncouplers) into the inner mitochondrial membrane" should be added as an MIE. Such partitioning to the cellular target is a chemical-specific property reflecting aspects of absorption, distribution, metabolism, and elimination (ADME). Unlike the chemical-specific IPCS Mode of Action framework, AOPs do not include ADME considerations in the AOP. Rather the AOP really starts with a biological perturbation of the target (i.e., after the stressor has arrived at its target). Thus, we feel that uncoupling (whether lumped or split) is the appropriate starting point for the AOP. A mechanistic description of the toxicology may include that partitioning (which is why we describe it in the text even though it is not explicitly represented as a KE), however, key events are intended to represent major, measurable, milestones or way points along a progression to the adverse outcome. The aim is not to include a KE that represents every detailed step, but to identify "check-points" along the path to adversity that one could use to increase confidence that an outcome linked to the MIE or an early KE is actually going to occur. We would note for example that partitioning of a chemical to the site of expressed estrogen receptors, androgen receptors, thyroid peroxidase enzymes, etc. is not part of established AOPs for which activation of those receptors or inhibition of those enzymes is the molecular initiating event. Therefore, we feel it is more consistent with the guidance and precedents associated with the framework to NOT include partitioning of the stressor as an initiating event.

4. **Linking decreased growth to decreased population trajectory:** Next, with regard to the authors' response to our comment number 16 in the initial review, we must say that we still do not completely understand the reasons for the authors' decision to not include population decline as a second AO in their AOP, despite the fact that, as they state themselves, "that particular linkage has been long accepted within the field of ecotoxicology to the point where it is accepted as canonical knowledge." If this is "canonical knowledge" indeed, then why wouldn't the authors acknowledge this and add the second AO? In other words, we were wondering if there are no other AOPs in the AOPwiki currently that have already sufficiently characterized the link from the AO "growth, decrease" to the AO "population, decline"? And if yes, could the authors "reuse" this particular relationship in their own AOP? After all, the possibility to "reuse" the already-existing KEs and KERs is one of the main advantages offered by the AOPwiki, hence we feel that the authors could have made a conscious effort to promote this practice.

Response: There is one established KER in the AOP-Wiki that links "decreased growth" to "decreased population trajectory" (Relationship 2169). However, at present that page is completely unpopulated. There are no AOPs linked to it. There is no description or assembly of evidence. We are not necessarily opposed to adding this relationship to our AOP and populating the page in the AOP-Wiki. However, as the link between growth and potential population-level impacts is considered canonical knowledge in the field of ecotoxicology we do not feel it is necessary or within the scope of our current project, to do a comprehensive assembly of primary evidence supporting this linkage. We could offer to site a couple text books and/or regulatory precedents, but we do not feel a detailed review of literature supporting this connection is needed, as effects on growth are already well accepted as being of regulatory relevance. We note that there is a paper on "Pragmatic AOP development" that is in preparation that makes the exact point that for canonical knowledge widely accepted in the field, there is no need to employ a systematic assembly of evidence to support a KER. Similarly, the guidance on AOP development states "…. it is recognized that there may be cases where the biological relationship between two KEs is very well established, to the extent that it is widely accepted and consistently supported by so much literature that it is unnecessary and impractical to cite the relevant primary literature. Citation of review articles or other secondary sources, like text books, may be reasonable in such cases." (page 40 of the Users' Handbook) - Thus, if we were to include this KER, we would propose to only include citation of a few such sources. There is not necessarily evidence for this relationship that is specific for uncouplers and the upstream events in the present AOP, thus we feel more extensive development of this KER is tangential to our primary project objectives.

Alternatively, we could add a few more sentences in the report to state that population decline is a potential higher level AO linked to growth inhibition, but the relationship warrants further development for empirical support.

5. **Environmental Bias:** Lastly, with regard to the authors' response to our comment number 15 in the initial review, we would like to share that we still feel that the descriptions accompanying this AOP continue to have a strong environmental bias, while human health applications are less visible. This is okay in the end, as this simply reflects the authors' main expertise. We, however, suggest that the authors consider adding a clear upfront statement acknowledging this and explicitly postulating that this AOP does have both the environmental and human health application.

Response: Agree and we will add a few more sentences to emphasize that the AOP is for both human and eco.

6. **Minor edits suggested:** Page 3, line 102: insert “on” before “growth” to have “focuses on growth inhibition”; Page 6, line 221: delete “that” before “not every”; should become “There can also be large tissue-specific effects and not every cell type is equally susceptible […]”

Response: Agree and we will revise these accordingly.

Reference

Ives C, Campia I, Wang R-L, Wittwehr C, Edwards S. 2017. Creating a Structured Adverse Outcome Pathway Knowledgebase via Ontology-Based Annotations. Applied In Vitro Toxicology 3:298-311. DOI: 10.1089/aivt.2017.0017.

Third review

Reviewers' answer to authors' responses to the second review round for AOP 263.

July 22, 2021.

Dear Authors, Dr. Song and Dr. Villeneuve,

Dear Editor Knapen,

We appreciate the authors' consideration and detailed responses to the remaining comments we've provided in the second review round. We are pleased to inform that we support the way forward proposed by the authors (see also our specific answers to some of the authors' responses below). Hence, we recommend that, after appropriate changes to the manuscript and the AOP-wiki have been made, the manuscript be accepted for publication in ET&C. We thank the editor and the authors for organizing and carrying through this collaborative review process, which provided a valuable learning experience for us as well.

Sincerely,

Ksenia Groh
David Dreier
Joel Meyer
Terry Schultz

Answer to response #1: We appreciate the detailed discussion provided by the authors in response to our remaining request to consider splitting the MIE "coupling of OXPHOS, decrease" into two separate KEs. We understand the authors' position and arguments against the complete splitting, particularly related to the lack of experimental approaches to directly measure the uncoupling action by methods other than dissipation of PMF. Consequently, we support the authors' suggestion to implement the "Event Components" approach and "keep the lumped MIE term of 'decreased coupling of OXPHOS', but differentiating 'diffusion across the IMM and transport protons out (uncoupling action)' and 'dissipation of PMF' as two event components associated with this MIE." This solution appears to fully address the anticipated needs, as it provides the possibility to connect to other AOPs and/or upgrade to separate KEs in the future, and at the same time allows keeping the originally developed AOP structure and making the best use of the evidence already collected by the authors.

Answer to response #3: We thank the authors for the detailed discussion of this point as well and concede to their decision to not include partitioning of the stressor as an initiating event.

Answer to response #4: We thank the authors for the provided explanations and suggest to adhere to "alternative solution" they've proposed, namely to "add a few more sentences in the report to state that population decline is a potential higher level AO linked to growth inhibition, but the relationship warrants further development for empirical support."

Responses to reviewers #3 - AOP 263 report

Dear Editor and Reviewers,

Please find below a list of revisions to the manuscript and AOPWiki. We hope the revised AOP report fulfills the requirements for publication in Environmental Toxicology & Chemistry.

Sincerely,
Dr. Song and Dr. Villeneuve
August 5, 2021

❑ List of revisions

AOP report

1. **Line 90-91**, added "These AOPs are considered highly relevant and applicable to both human health and ecological risk assessments."

2. **Line 102**, added "on"

3. **Line 104-107**, added "Moreover, it is biologically plausible that population decline is a potential higher level AO linked to growth inhibition, but the relationship warrants further development for empirical support and will not be included in the current AOP described herein."

4. **Line 109-110**, revised to "partitioning of protonophores (uncouplers) into the inner mitochondrial membrane is known to uncouple OXPHOS, leading to dissipation of proton motive force and subsequent reduction in ATP synthesis."

5. **Line 127-134**, revised to "binding of protons in the inter membrane space, transportation of protons across the inner mitochondrial membrane (uncoupling action) and dissipation of protonmotive force. The first two actions are considered difficult to measure, whereas the third can be proportionally indicated by mitochondrial membrane potential, proton leak and/or oxygen consumption rate. The three intermediate events are therefore considered as Key Event Components (Ives 2017) to support the MIE in the AOPWiki. It should be noted that "dissipation of protonmotive force" is an important event that is relevant to many other AOPs and has a great potential to be considered as an independent key event in the future with the evolvement of knowledge and analytical technology."

6. **Line 222**, deleted "that"

7. **Line 282**, added "(including human)"

8. **References**, added "Ives C, Campia I, Wang R-L, Wittwehr C, Edwards S. 2017. Creating a Structured Adverse Outcome Pathway Knowledgebase via Ontology-Based Annotations. Applied In Vitro Toxicology 3:298-311. DOI: 10.1089/aivt.2017.0017."

AOPWiki

9. **Main page, Abstract**, revised to "This AOP is of high regulatory relevance, as it is considered applicable to both human health and ecological risk assessments."

10. **Event 1446 page, Key Event Component**: Added 3 KE components to support the MIE

Process	Object	Action	GO ID
proton binding	mitochondrion	increased	GO:1901691
oxidative phosphorylation uncoupler activity	mitochondrion	increased	GO:0017077
regulation of mitochondrial membrane potential	mitochondrion	decreased	GO:0051881