Scientific review report for AOP 360 on Chitin synthase 1 inhibition leading to mortality

Reviewers comments and authors responses

This review concerns the AOP publication authored by Simon Schmid, You Song and Knut Erik Tollefsen and submitted to Environmental Toxicology & Chemistry. The reviewed materials consisted of a snapshot of the AOP360 "Chitin synthase 1 inhibition leading to mortality" captured from the AOPwiki (https://aopwiki.org/aops/360) along with the accompanying manuscript titled "AOP Report: Inhibition of Chitin Synthase 1 Leading to Increased Mortality in Arthropods".

General Reaction: AOP 360 Wiki Entry and Manuscript

The Review Team (Ankley, Houde, Poynton) has completed evaluation of the package submitted by Schmid et al. describing an AOP concerning effects of inhibition of chitin synthase on arthropod survival. The review included consideration of both the AOP 360 "snapshot" from the AOP Wiki and the paper submitted for consideration for publication in *Environmental Toxicology and Chemistry*. The Team is strongly supportive of the development/evolution of opportunities to publish/highlight AOP content in the open peer-reviewed literature and commend Schmid et al. on their efforts in providing in inaugural effort in this area.

All three reviewers evaluated both documents. To simplify the author's task in terms of revising the journal article, detailed corrections/suggestions/comments have been incorporated into a single track-changes Word document that accompanies this file. Input on the manuscript was provided relative both to technical issues and editorial recommendations designed to enhance readability by the type of general audience likely to read journal front matter, but not necessarily the Wiki entry.

In terms of review of the AOP Wiki content, there was a far lesser emphasis by the Review Team on editorial aspects of the presentation. Rather, the snapshot was assessed as to technical merit/completeness and clarity of presentation for an audience likely to be more aligned with the nature and requirements of AOPs than the typical reader of a featured journal article. Overall, the Team felt that the Wiki entry was relatively robust from a technical perspective, and generally clearly presented. A few specific comments are included in the pdf file and should be considered during revisions[1].

Below is a summary of higher-level technical and editorial issues concerning the Schmid et al. submission(s). Most of these comments deal with the journal article, although some clearly are applicable to both documents.

General Comments and Recommendations

(1) The name of the molecular initiating event "Increase chitin synthase 1 inhibition" seems unnecessarily complicated and, perhaps, inaccurate. The proposed name implies that there is a baseline inhibition that is being increased by a stressor, which doesn't seem to be the case. Perhaps a more accurate title would be "Decrease chitin synthase 1 activity"?

[1] These comments have been reported and addressed in the tables titled 'Specific Responses to Reviewers' Comments on the Manuscript' and 'Specific Answers to Reviewers Comments on the AOP-Wiki Page' on pages 9-19 of the present report.

(2) Section 1. Introduction and Background and 3. Scientific Evidence Assessment are over the maximum word limits and should be shorten. This is a concern because it is a relatively straightforward AOP and future, more complex AOPs would likely need even more space. As a precedent, this paper should present the AOP in a more focused manner and limit information that is not directly related to the AOP.

(3) Figure 1 might be a useful addition to the journal article in the context of providing an overview of basic biochemistry of the system. However, the figure is pretty "acronym heavy", and many different facets of it are not well described either in the text or figure legend, e.g., the meaning of differently colored nodes, the significance of (one) compensatory feedback response, etc. Please see the Word file.

(4) Lines 105-110 in the paper are confusing. It seems as if a determination of CHS activity is, in fact, a direct measure of the proposed MIE rather than a KE option. And, cuticular chitin content is a logical KE1 pursuant to impacts on the MIE. So it seems that both are KEs in the AOP (the MIE is considered a type of KE). Some clarification would be useful here as to what the authors' intent.

(5) Lines 109-113 in the paper describe the KE "Increase, premature molting." This key event is poorly described and it difficult for the reader to understand how it might be observed or measured. Please define the "variety of effects" that characterize pre-mature molting. Some examples are provided in lines 165-169, which should be explained earlier when pre-mature molting is introduced.

(6) The Review Team questions how the proposed AOP network is presented in the text of the draft manuscript (Lines 117-131). Many readers will not be aware exactly what an AOP network is, so some additional basic background would be needed to introduce the concept (and its utility). Also, the current text is too brief—especially without a supporting figure—to really visualize what comprises the network. There is some question as to whether the best strategy for the paper would be to increase description of the network (and add a figure) or to reduce the amount of text currently devoted to describing it. Part of the challenge in deciding which route to go is that it is not entirely clear how knowing more detail about the network enhances description/presentation of AOP 360.

(7) Multiple examples are given in section 3.1 which are interesting but take much space. We suggest to transfer these examples/species and related references in a column in Table 1 to make the text shorter.

(8) Section 3.3: Empirical evidence: The reviewers felt that results from knock-out (KO) studies included in the essentiality of the KEs could be used to support a empirical evidence rating of "moderate." Many of the studies referenced in lines 160-176 of the manuscript appeared to have measured multiple endpoints that could support temporal concordance of the KERs. We agree that dose response and response-response data is lacking and so we do not support a rating of "high."

(9) There are aspects of the following comments concerning evaluation and description of taxonomic domain of applicability that are germane both to the Wiki entry and the journal article. From a broad perspective, evaluation of taxonomic domain of applicability of an AOP involves more than just an analysis of cross-species structural conservation of the (protein) MIE. While this is a logical step, and SeqAPASS is an excellent tool supporting the evaluation, there are other components

contributing to analysis of taxonomic domain of applicability, including (a) evaluation of functional conservation of the MIE (e.g., through comparative in vitro assays); (b) determination of cross-species conservation of KEs other than the MIE; (c) consideration of general cross-taxa conservation of the role of chitin synthesis relative to molting (i.e., knowledge of basic arthropod physiology); and (c) evaluation of cross-species commonality in apical responses to stressors that ostensibly would affect this pathway (e.g., pesticides). All these considerations could contribute to a weight-of-evidence assessment of the taxonomic domain of applicability of a given AOP. At present, emphasis in the Wiki entry concerning this cross-species applicability is largely only on the SeqAPASS structural analysis.

In the manuscript, description of the taxonomic domain of applicability essentially describes a three-level SeqAPASS-based structural analysis. While this is certainly an appropriate and useful addition to the paper, the section is written assuming that the reader not only knows what SeqAPASS is conceptually, but also details as to how an analysis is done. This is likely to be true for only a relatively small number of readers. In the track-changes version of the paper the Review Team provides several editorial suggestions as to the nature and conduct of the SeqAPASS analysis that should make the section a bit more interpretable for an average reader. However, the entire section was not edited in this manner (basically editing stopped when the description moved to Level 3), so the authors need to do some additional revision.

(10)The final section of the paper, “Applications of the AOP”, needs some significant attention. It is quite likely that this will be the section of most interest to many readers, especially those involved in risk assessment/management (i.e., key clients/consumers of AOP content). The current section mentions several different directions/applications for the AOP, but in such a brief/cursory manner that there is no clear take-home message. And, concepts are introduced here for the first time in the paper (e.g., IATA) but not described to a degree that an uninitiated reader would know to what the authors are referring to in terms of AOP use. Basically, the concluding section to the paper lacks a core theme around which the “so what” issue can be addressed. The Review Team acknowledges that this sort of synthesis section can be a challenge to write, but it seems critically important to ensuring that the paper is successful.

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Dries Knapen
Environmental Toxicology and Chemistry (ET&C)

Oslo, February 26, 2021

Dear Dries

Thank you for giving us the opportunity to resubmit a revised version of our manuscript entitled "Inhibition of Chitin Synthase 1 leading to Increased Mortality in Arthropods" for reconsideration for publication in *Environmental Toxicology and Chemistry*. We also thank the three reviewers (Gerald Ankley, Magali Houde, and Helen Poynton) for a very thorough review and numerous very helpful suggestions on the manuscript. We truly believe that the changes suggested by the reviewers significantly improved the manuscript and the AOP-Wiki page (https://aopwiki.org/aops/360).
You can find our answers to the reviewers' general suggestions below, as well as specific responses to each of the reviewers' comments on both the manuscript and the AOP-Wiki page. Changes in the revised manuscript are highlighted in yellow.

We look forward to hearing back from you!

Sincerely,

Simon Schmid (PhD Candidate, MSc)
Section for Ecotoxicology and Risk Assessment
Norwegian Institute for Water Research (NIVA)

Responses to Reviewers' General comments: AOP 360 Wiki Entry and Manuscript

(1) The name of the molecular initiating event "Increase chitin synthase 1 inhibition" seems unnecessarily complicated and, perhaps, inaccurate. The proposed name implies that there is a baseline inhibition that is being increased by a stressor, which doesn't seem to be the case. Perhaps a more accurate title would be "Decrease chitin synthase 1 activity"?

We thank the reviewers for this suggestion. Indeed, the name of the molecular initiating event "Increase, chitin synthase 1 inhibition" seems overly complicated and suggests a baseline inhibition of the enzyme. We therefore changed the name of the MIE to "Inhibition, chitin synthase 1" in the manuscript and in the AOP Wiki.

(2) Section 1. Introduction and Background and 3. Scientific Evidence Assessment are over the maximum word limits and should be shorten. This is a concern because it is a relatively straightforward AOP and future, more complex AOPs would likely need even more space. As a precedent, this paper should present the AOP in a more focused manner and limit information that is not directly related to the AOP.

We understand the reviewers concerns about the length of the sections 1. Introduction and Background and 3. Scientific Evidence Assessment and appreciate the very helpful suggestions of the reviewer team on how to shorten the sections. We made several changes in order to shorten the manuscript and present AOP 360 in a more focused way. Specifically, we removed information about upstream processes which are not essential for the presentation of AOP 360 in the Introduction and Background section.
In the Scientific Evidence Assessment section, we removed the common and Latin species names under point 3.1 (Essentiality of KEs). For detailed information on studies we refer the reader to Table S1, which gives a very detailed overview on the studies used for the assessment of essentiality of KEs.
Under point 3.2 (Biological plausibility of KERs), we removed text that described the transcriptional regulation of CHS-1. We generally shortened section 3.3 (Empirical Evidence of KERs) and are now stating that information on stressor-response and response-response relationship is sparse in a more general way rather than for each KER.
In section 3.4 (Chemical applicability domain), we highly appreciate Gerald's edits to the text which makes it shorter.
We felt that section 3.5 (Taxonomic applicability domain) was excessively long. We now give a short introduction on how the SeqAPASS tool works and briefly present the most important results and refer to the supplementary information, to where the more detailed description of the approach and the results has been moved.
Editorial changes suggested by the reviewers have been accepted as they improve the flow of the text and make it shorter at the same time.

(3) Figure 1 might be a useful addition to the journal article in the context of providing an overview of basic biochemistry of the system. However, the figure is pretty "acronym heavy", and many different facets of it are not well described either in the text or figure legend, e.g., the meaning

of differently colored nodes, the significance of (one) compensatory feedback response, etc. Please see the Word file.

We changed the figure according to the suggestions of the reviewers to make it clearer. Namely, we explained what the colors of the boxes mean and removed the feedback loop as its relevance for the AOP is not clear. Further, the figure was moved to the supplementary information because it depicts many upstream processes that are not essential for the presentation of AOP 360.

(4) Lines 105-110 in the paper are confusing. It seems as if a determination of CHS activity is, in fact, a direct measure of the proposed MIE rather than a KE option. And, cuticular chitin content is a logical KE1 pursuant to impacts on the MIE. So it seems that both are KEs in the AOP (the MIE is considered a type of KE). Some clarification would be useful here as to what the authors' intent.

We deleted the lines 105-110 and agree with the reviewers that this might confuse many readers. We agree that

(5) Lines 109-113 in the paper describe the KE "Increase, premature molting." This key event is poorly described and it difficult for the reader to understand how it might be observed or measured. Please define the "variety of effects" that characterize pre-mature molting. Some examples are provided in lines 165-169, which should be explained earlier when pre-mature molting is introduced.

We moved the mentioned examples to Section 1. (Introduction and background)(Lines 53-57) and mention them in Section 2 (AOP description) on lines 79-82. Suggestions on how to measure this endpoint are given in BOX2. The KE description in the AOP wiki has been populated with examples as well.

(6) The Review Team questions how the proposed AOP network is presented in the text of the draft manuscript (Lines 117-131). Many readers will not be aware exactly what an AOP network is, so some additional basic background would be needed to introduce the concept (and its utility). Also, the current text is too brief—especially without a supporting figure—to really visualize what comprises the network. There is some question as to whether the best strategy for the paper would be to increase description of the network (and add a figure) or to reduce the amount of text currently devoted to describing it. Part of the challenge in deciding which route to go is that it is not entirely clear how knowing more detail about the network enhances description/presentation of AOP 360.

We decided to only mention the network briefly, so the reader knows that it exists (Lines 87-90). We removed most of the text regarding to the network as it is not crucial to the description of AOP 360 and the manuscript should focus on this AOP. Further, providing more details on the network does not enhance the presentation of AOP 360.

(7) Multiple examples are given in section 3.1 which are interesting but take much space. We suggest to transfer these examples/species and related references in a column in Table 1 to make the text shorter.

Thanks for this suggestion. We removed the species names in the text and limited the number of references to a couple of representative ones. We refer the reader to Table S1 for a very detailed compilation of studies used for the assessment of Essentiality of KEs as we intended to give a short summary on the assessment of Essentiality in Table 1.

(8) Section 3.3: Empirical evidence: The reviewers felt that results from knock-out (KO) studies included in the essentiality of the KEs could be used to support a empirical evidence rating of "moderate." Many of the studies referenced in lines 160-176 of the manuscript appeared to have measured multiple endpoints that could support temporal concordance of the KERs. We agree that dose response and response-response data is lacking and so we do not support a rating of "high."

Thanks for this suggestion. We added the information to the manuscript and referenced the studies that measured all endpoints along the AOP (Lines 214-217). We also added the studies to Table S2. These studies are now also mentioned in the AOP-Wiki.

(9) There are aspects of the following comments concerning evaluation and description of taxonomic domain of applicability that are germane both to the Wiki entry and the journal article. From a broad perspective, evaluation of taxonomic domain of applicability of an AOP involves more than just an analysis of cross-species structural conservation of the (protein) MIE. While this is a logical step, and SeqAPASS is an excellent tool supporting the evaluation, there are other components contributing to analysis of taxonomic domain of applicability, including (a) evaluation of functional conservation of the MIE (e.g., through comparative in vitro assays); (b) determination of cross-species conservation of KEs other than the MIE; (c) consideration of general cross-taxa conservation of the role of chitin synthesis relative to molting (i.e., knowledge of basic arthropod physiology); and (c) evaluation of cross-species commonality in apical responses to stressors that ostensibly would affect this pathway (e.g., pesticides). All these considerations could contribute to a weight-of-evidence assessment of the taxonomic domain of applicability of a given AOP. At present, emphasis in the Wiki entry concerning this cross-species applicability is largely only on the SeqAPASS structural analysis.

In the manuscript, description of the taxonomic domain of applicability essentially describes a three-level SeqAPASS-based structural analysis. While this is certainly an appropriate and useful addition to the paper, the section is written assuming that the reader not only knows what SeqAPASS is conceptually, but also details as to how an analysis is done. This is likely to be true for only a relatively small number of readers. In the track-changes version of the paper the Review Team provides several editorial suggestions as to the nature and conduct of the SeqAPASS analysis that should make the section a bit more interpretable for an average reader. However, the entire section was not edited in this manner (basically editing stopped when the description moved to Level 3), so the authors need to do some additional revision.

We acknowledge that the assessment of taxonomic applicability also should have other components than the SeqAPASS analysis. Therefore, we referred to the basic arthropod physiology, and state that all arthropods need to molt in order to develop and hence are dependent on the synthesis of chitin, which makes it highly likely that the AOP is relevant for the whole phylum of arthropods. We also looked at the conservation of KEs in the assessment in the wiki, although data on effects are limited. We believe that this information can take the emphasis away from only SeqAPASS analysis, which remains an important component of the assessment. We also briefly mention the beforementioned components in the manuscript.

In regard of the length of the section, we had to move more detailed information on the approach and results of SeqAPASS analysis to the supplementary information (we still appreciate the reviewer's edits!). In the manuscript, we now briefly introduce the different levels of alignment (Lines 264-267) and the most important results (273-280) and refer the reader to the supplementary information for details. Additionally, we moved Figure 4 (now Figure S2) depicting percent similarities of SeqAPASS Level 1 and Level 2 analysis to the supplementary information as not all readers may be familiar with the tool and the figure may therefore not be easily interpretable for all of the readers.

(10) The final section of the paper, "Applications of the AOP", needs some significant attention. It is quite likely that this will be the section of most interest to many readers, especially those involved in risk assessment/management (i.e., key clients/consumers of AOP content). The current section mentions several different directions/applications for the AOP, but in such a brief/cursory manner that there is no clear take-home message. And, concepts are introduced here for the first time in the paper (e.g., IATA) but not described to a degree that an uninitiated reader would know to what the authors are referring to in terms of AOP use. Basically, the concluding section to the paper lacks a core theme around which the "so what" issue can be addressed. The Review Team acknowledges that this sort of synthesis section can be a challenge to write, but it seems critically important to ensuring that the paper is successful.

In the last section, we kept the first part (Lines 356-365), it briefly introduces why arthropods are important for the environment and why it is important to have an AOP for CHS-1 inhibition for susceptible non-target species.
As the reviewers suggested, we selected three core themes which we developed more in-depth as it has been done before. As knowledge of chemicals directly interacting with CHS-1 is limited, guiding screening approaches for chemicals that do so is an obvious application of the AOP. We elaborated on how this could be done and how the AOP might help identify assays for further testing.
In the last paragraph we introduce how a qAOP might be of use in the estimation of safe levels of chemicals interacting with CHS-1.

Specific Responses to Reviewers' Comments on the Manuscript

Reviewer # 1

Line	Comment	Response
23	How are endocrine disruptors defined here, and how is that definition different than inhibitors of enzymes? For example fadrozole, which inhibits enzymes involved in steroid synthesis in vertbrates, is considered an endocrine disruptor.	We specified which enzymes are meant (Line 20-22). Chitin synthesis inhibitors only resemble endocrine disruptors by interfering with molting which is under endocrine control. Their mode of action, however, is purely non-endocrine as they have no influence on the endocrine system (unlike e.g. aromatase inhibitors in vertebrates).
38	Identify abbreviation at first use	Done. (Line 41)
77	Should state what the different colors of the figure components mean	We stated this in the figure description (Figure S1)
87-88	Unclear what this means or the context of the statement relative to AOP under consideration	We agree and deleted the text in the figure description and removed the feedback loop in the figure (Figure S1).
99	'increase in inhibition' suggests some sort of baseline or normal inhibition; perhaps the term here is just inhibition?	We agree that there is no baseline inhibition and therefore changed the name of the MIE to "Inhibition, Chitin synthase 1".
106-107	This statement is likely to confuse many. The AOP MIE is inhibition of of CHS which is the same as inhibition of chitin synthesis activity, correct? This is, by definition, a KE. The next KE would be a change (decrease) in chitin content. Basically I think that both these processes are in the AOP.	We agree, the decrease of activity of CHS-1 is the functional outcome of the inhibition of CHS-1 (stated on Line 71-72) and can therefore be considered equivalent. We deleted the sentence to not confuse readers.
115	'lumping' certainly gets the point across, but is not very techncial. Perhaps 'combining'?	Done (line 85).
117	I wonder if a simple figure depicting the network would be useful—a little hard to follow based solely on the text. Or, if there is a desire to limit length of the paper, the AOP network discussion could be largely dropped other than noting that the AOP described in the paper will be down the line incorprated into an AOP newtwork.	We decided to drop the discussion on the network and focus on AOP 360. We just briefly mention the network so the reader knows that AOP360 is part of it (Line 86-90).
120	Why capitilized?	This part was deleted.

128	Will need to add citation to references section as per ET&C required formating. Society for the Advancement of Adverse Outcome Pathways (SAAOP). 2016. Welcome to the Collaborative Adverse Outcome Pathway Wiki (AOP-Wiki). Available from: https://aopwiki.org/	Done, also for other web pages. The citation style should now be appropriate for ETC.
133	As noted previously, the MIE title 'Increase CHS inhibition' implies that there is a baseline inhibition to increase from. Would the more accurate descriptor be 'Decrease (or inhibit) CHS activity'?	We changed the name of the MIE to "Inhibition, Chitin synthase 1" as "Inhibition" suggests an interaction of the enzyme with a chemical.
AOP ID BOX	Consider modifying MIE title as per above	Done.
148-149	Statement seems rather 'stand alone', with no context as to how this was decided. Maybe the sentence needs to be modfied by adding a phrase like '*As described in greater detail below* the overall confidence...' Basically there is no mention here of the WoE concept...	Thanks! We included a short summary of the WoE evaluation for this particular AOP here (Line 108-115). The mentioned sentence now fits in nicely as last part of this short summary.
160-162	Here and later, I don't know that it is necessary to include the Latin names of the organisms given that they are not, techncially, used for experimentation descrbed in the paper.	We agree, deleted the names of the species and only refer to "insects" now. Interested readers can consult Table S1 for details on species and studies used for the evaluation of essentiality.
172-178	If there is a desire to shorten text, these more detailed examples could be excluded—the prior sentence captures the essence of the studies	We moved the examples to the background section (Lines 52- 56).
197-200	While this compensatory loop could well exist I am not sure how it is related to evaluation of essentiality? Could be removed.	Done, removed the sentence.
214-219	I wonder whether this is a more elaborate treatment of plausibility of an AOP based on inhibition of CHS that is needed? That is, does plausibility in this case necessarily include regulation upstream of the MIE?	We agree, this is not necessary. Deleted the paragraph.
290-291	Sentence seems incomplete to me	Deleted the sentence.
295-297	How were the expsoures conducted in other studies? Diet, injection? This statement leads to conjecture about ADME variaions which	Usually they were injected, but we deleted the sentence, because this

	may be more detail than can be accomodated in the paper... Perhaps could be dropped?	may indeed be too detailed for an AOP report.
300	Concept of qAOPs has not been touched on in the paper. Might need a slight expansion of the concepot in order for this sentence to make a lot of sense to the reader.	Thanks for this comment. We included a brief introduction to the qAOP concept here (Line 222-224).
302	Not everything on this table is mentioned in the text, e.g., detection method and target, which may add some confusion. Maybe these two columns should be removed? At a minimum need to define detection method and target... Or, I wonder if this table could be moved to the SI?	The two columns were removed, as suggested. Table 1 summarizes the evaluation of essentiality of KEs in the manuscript and for details, the reader can consult Table S1.
304	Good summary table, but... Although components of weight of evidence (WoE) assessment were presented, the term/concept was not actually dicussed in the text. Switching to this terminology in Table 2 may confuse readers. Somewhere there would need to be a few lines disucssing combination of essentialty, plausibility and empirical evidence into a WoE assessment for this specific AOP.	A short introduction to WoE was added to the beginning of the section, with a short summary of the WoE for this specific AOP (Line 108-115).
324-325	Could be removed—specific use on one is getting a bit more specific than needed...	Done.
334-336	This last sentence and reference could be removed. Not really all that gemane to evaluation of the AOP chemical space.	Removed the sentence and associated references.
340	This section is written assuming that the reader is familiar with the concepts/terminology of SeqAPASS. This usually will not be the case. I made several editorial changes/suggestions to try to address this but stopped on line 366 because I felt that my changes were so extensive that I was totally rewritng the text. So, if there is a desire to pursue description of the Level 3 SeqAPASS analysis, the authors should consider rewrting this (lines 367-397)	Thanks for the editing! As the section was too long, we decided to just briefly present the approach and results from the SeqAPASS analysis and move the more detailed information on methodology and results to the SI.

	in a manner more accessible to readers not familiar with the tool. Basically, the analysis and interpretations are reaonable but only someone with intimate knowldege of SeqAPASS would understand what is going on.	
369-370	Stopped editing this section here.	
417-424	Figure won't be easily interpretable for most readers.	We understand that readers that are not familiar with SeqAPASS might have problems interpreting the figure. We therefore moved the figure to the SI (Figure S2)
427	This section covers a lot of possibilities but perhaps not in enough detail to let the reader know how having an AOP for CHS inhibition would be helpful. There are many concepts/possible uses that are rather run-together in a manner likely to be confusing to the reader. Perhaps it would be good to select two or three uses and fully develop these rather than trying to cover as much ground as currently is done? For example, it seems to me that the main utlity would be to support the development of computational approaches and/or short-term in vitro methods to rapidly and cost effectively screen for chemicals that inhibit CHS. The AOP provides a basis both for method development (what to predict/measure) in the context of making linkages to apical effects relevant to risk assessors. Maybe it would be good to discuss specifc regulatory programs/mandates where this would be needed, e.g., REACH in Europe or pesticide registration in the US? Another practical example of application of the AOP would be programs focused specifically on protecting nontageted species of concern like pollinators.	Thanks for these suggestions. The previous version of this section was indeed very broad. We basically rewrote the whole section. We now focus on 1) the screening approach, providing examples on how such a screening might be done 2) on how the AOP might be able to guide targeted testing and 3) on how KERs might be quantified and used in risk assessment.
437	Not clear what 'missing link' refers to	Removed.

440	Unclear	Removed.
445-447	Seems like this is a different use than developing screening tools—talking about chemical discovery/pesticide development here perhaps?	Removed.
449-451	Certainly a use of SeqAPASS, but maybe not the AOP?	Removed.
451-453	Reference to this likely will confuse many readers without further context.	We agree, removed this.
454-458	Concept of qAOPs hasn't previously been touched on, although it is reasonable that this qualitative AOP could lead to a quantitaive AOP. Also, the term PODs is rather 'jargony'. Perhaps could stick with exceedence of possible effects concentrations?	We give a brief intro to the qAOP concept earlier in the manuscript (Line 222-224).
459-462	Many readers will not know what IATA refers too and, while the concepts certainly would be understood by most, this is likely too brief to be very informative.	We agree and removed the concept of IATA.

Reviewer # 2

Line	**Comment**	**Response**
1	This section exceeds the maximum 800 words	We shortened the text of this section to meet the specifications of the author guidelines.
7	I suggest adding '...such as insects and aquatic invertebrates	Thank you! Added this.
10	What are 'benefical arthropods'? As opposed to what exactly? I suggest using the term 'non-target'	We now use the term 'non-target'.
10-12	Is this sentence essential? Given the exceeding number of words for this section, maybe consider deleting.	The sentence was removed.
15	Said on L18-19	Removed this.
18	Already defined on L16	Removed this.
36	L36 Insert paragraph break here? The cuticular chitin...	Done.
56	A little repetitive	Agree, removed the sentence.
68	As also discussed further below maybe a word could be added here to briefly explain the ecdysis motor program (i.e.…)	Thanks for the suggestion, we added a brief explanation on the ecdysis motor program on Line 35-37.

77	Interesting figure. Include (Mg^{2+}) next to magnesium in the caption. To keep in the main text or transfer to SI?	We included (Mg^{2+}) next to magnesium in the figure caption and moved the figure to the SI.
117	I agree with a network figure that could be added to SI	We decided to focus on AOP 360 in this report. We dropped most of the discussion of the network and now only briefly mention it so the reader knows that there are other AOPs associated with AOP 360.
141	2500 word limitt – currently > 3000	We shortened the text of this section to meet the specifications of the author guidelines.
155-157	Do we need to know the related species/animal groups studied for these stressors?	We added relevant taxa here (Line 121).
161-169	Could some of these examples/species and related references be added in a column in Table1 to make the text shorter?	We decided to delete these species names. Table S1 gives a very detailed overview over the studies used for the assessment of essentiality. We refer the reader to Table S1 in this section and in the caption of Table 1 for details.
172-178	Or add to table 1?	See response above.
178	Based on results presented above and in Table1? see comments above	We added the suggested info at the end of the sentence.
182	Again, are species important to indicate?	We added relevant taxa here (Line 135).
290-291	Delete the last part of the sentence?	Deleted the whole sentence.
302 (Table 1)	Could species and related references be added in a column to shortern the text?	Our intend with Table 1 was to give a short summary of the evaluation of essentiality. Teble S1 gives a very detailed overview of all studies used for the evaluation. Therefore we refer the reader to Table S1 for detailed information.
302 (Table 1)	Define RNAi	This column of the table was removed. However, we defined RNAi on Line 125.
304	The term WoE has not been defined in the text (nor the table).	Added a brief introduction to the WoE concept to the beginning of the section, along with a short summary of the overall WoE evaluation for this specific AOP.
307	Gary's editing in this sub-section does make the text shorter and easier to read.	We agree and accepted the changes.

350	A word to present the three levels of analysis for a better understanding of the text to come?	Thanks for this suggestion. We added a short description of the three levels on Line 265-268.
374-377	This sentence should be rephrased for clarity	Rephrased this sentence. (The text is now in SI)
383-389	This section needs to be better introduced to the reader. What do these acronyms stand for?	We moved this text to the SI. The protein motifs used for the level 3 SeqAPASS analysis re presented in a table there to present them in a clearer way.
419	The red point should probably be next to the name below the axis	Added this to the figure (Figure S2).
430-431	Very vague	We specified this with some examples (Line 302).
456	I agree with Gary, the very brief introduction of several new concepts, qAOP, POD and IATA, is confusing; too much new info in too few words.	We removed text that mentions concepts that have not been introduced befort (e.g. IATA and POD). And focused on a few core applications of the AOP.
475	I believe all authors have to be named, no use of et al. in the references for ET&C	Thanks for mentioning this and for the edits in the references! All author names are now displayed in the references.

Reviewer #3

Line	Comment	Response
1	Word count of almost all the sections far exceeds author guidelines and given that this is a relatively simple AOP, it seems that it should be below word limits. There are two ways the authors should address this: (1) make the language more concise. (2) remove information that is not directly relevant to the AOP. This second way is important not only to reduce the word limit, but also to focus the reader on the events and relationships of this AOP and not get confused by paralelle events that occur with in AOP network, but not directly part of this AOP.	Thanks for pointing this out. We decreased Word count by removing text that is not relevant for the presentation of AOP 360. In this way we present AOP 360 in a more focused way and significantly reduce the length of the text.
7	It is not clear what they are exposed to. Consider, 'Susceptible, non-target organisms exposed to chitin synthesis inhibitors may suffer . . . '	Thanks! We changed the sentence as suggested (Line 6).

49-50	This is not a complete sentence	Deleted this sentence and associated information about the regulation of CHS-1 and ecdysis. The information is now on line 33-38, together with information on the ecdysis motor program (brief introduction).
62-65	To better describe the observed effects, consider revising and adding: "These effects are observed in smaller animals following molting, which fail to survive subsequent molts (Chen et al. 2008) or animals being stuck in their exuviae (Wang et al. 2019) and ultimately dying due to insufficient food or oxygen intake (Arakawa et al. 2008; Camp et al. 2014; Song et al. 2017a)."	Thanks for the suggestion! We changed the text accordingly (Line 52-56)
77	Very nice figure, but it depicts many events outside the scope of the AOP, more within an AOP network. The figure could be useful as a supplemental figure. If a figure is desired, consider only events directly upstream CHS-1 and within the chitin synthesis pathway.	We moved the figure to the SI as suggested (Figure S1).
97	This event is sometimes referred to as 'inhibition of chitin synthase' and sometimes as 'increase in inhibition of chitin synthase' (i.e., in fig. 2 and AOP ID box.) The authors should use the first description as it is less confusing or if 'increase' or 'decrease' is a necessary descriptor for the key event, consider 'decrease in CHS-1 activity.'	We changed the name of the MIE to "Inhibition, Chitin synthase 1" in the manuscript and the AOP Wiki.
106-107	Reply, I agree that L105-L110 is confusing and that both are already represented in the AOP. I would consider deleting.	We deleted the mentioned part of the text.
110-112	It seems to me that a key event should not be 'a variety of effects.' This KE needs to be better defined to describe how it could actually be measured. Even if we agree that a variety of events can constituent one KE, these events are not well defined at any point in this AOP description.	We agree. We added some information to better describe this KE (line 78-86) and provide suggestions on how to measure it in BOX 2.
148-149	Agreed, it would be great to reference the OECD guidelines here for WoE categories and add short descriptions. This would also help with the interpretation of Table 2.	We added short descriptions on WoE categories along with a short summary of the results of the WoE evaluation for this particular AOP to help the

		reader with the interpretation of both Table 1 and Table 2.
172-178	These examples are important because they provide a description of what endpoints fall under 'premature molting.' However, it would be better to include a brief description of these endpoints earlier when premature molting is introduced as a KE. Suggested revised text was added above (line 62).	As suggested, we moved these examples to section 1. (Introduction and background) to introduce the KE "Increase, premature molting" (Line 52-56).
180	L180, start new paragraph.	Done (Line 134).
183	How was essentiality rated for decrease in cuticular chitin synthesis?	We included this information in the text (Line 137).
190	In the studies described here (lines 190-197), was cuticular chitin content measured? If not, I am not sure they can provide direct evidence for the essentiality of this KE. In this case, they should be removed or used to support a different aspect of the AOP.	The cuticular chitin content was measured in these studies, yes. We added the info in the text (Line 144-149)
214-219	I had a similar comment. While this is important for the AOP network, I think it should not be included in this particular AOP to keep it more focused.	We removed this part as it is not necessary for the assessment of biological plausibility for AOP 360.
232	Deleted 'respectively' here because there are many aquatic insects.	Thanks. We accepted this change.
251-253	The reviewers agreed that KO experiment data should be added to the empirical evidence section and could be used to support a 'moderate' level of emperical evidence. Line 160-70 provided examples of KO studies that measured mutliple endpoints that help to show temporal concordence. Also the study described on line 176 suggested that KO led to decreased cuticular content which in turn led to premature molting associated mortality.	We added the results form KO studies to support temporal concordance (Line 215.218). Detailed information on the studies used was added in Table S2.
276-278	This sentence is redundant, consider deleting.	Deleted.
289	Are new assays needed, or do assays exist, but have not been utilized in all studies? Box 2 below suggests that assays do exist.	Assays do exist, they just need to be used to link the effects. However, we felt that this sentence was abundant since it is a description of the evaluation of the AOP. Therefore, we deleted the sentence.
304	Yes, this is confusing. There are places in the text that state biological plausibility is high, empirical evidence was low, but in figure 2 it showed 'moderate' evidence for WoE. Without having read through the OECD	We added a short summary of the WoE evaluation at the beginning of this section (Line 108-115) to facilitate the interpretation of Table 2 for the reader.

	guidelines carefully, the reader will not understand why these are different and how they are related to each other. A summary of their relationships should be presented in the text.	
345-347	I really liked this approach, but I am wondering if the details of how it was done are too much for a AOP description. Perhaps these details could go into the SI with a simple overview of the results describing: Overall full sequence similarity, similarity across the catalytic domain, and presence of specific motifs.	We moved the more detailed description of the approach to the SI and briefly introduced the SeqAPASS tool and the results here (Line 263-268, 273-280).
402	This section should state the this AOP is relevant for all sexes.	We added this information (Line 295-296)
450	Which enzyme? Was the mutation in the CHS-1? If not, I do not think it is relevant to this AOP.	The mutation was found in CHS-1, yes. We removed this part to not confuse the reader.

Specific Answers to Reviewers Comments on the AOP-Wiki Page

Reviewer # 2:

Section	**Comment**	**Answer**
Abstract (AOP Page)	I'm not sure about the structure of this first paragraph. I suggest starting by citing the importance of molting and cuticle and then discuss chitin and CHS1.	Done, the structure was changed as suggested.
Background (AOP Page)	Good section, easy to follow	Thanks!
Background (AOP Page)	Should be made clear at the start that insects and crustaceans are part of this same phylum. Arhtropods (including insects, arachnids and crustaceans)... This information should also be added to the main manuscript.	Done. Changed this also in the manuscript.
Events (AOP Page)	Increase and inhibition is difficult to follow = decrease? or just inhibition?	Changed the MIE title to "Inhibition, Chitin Synthase 1"
Domain of Applicability (AOP Page)	Should the 'Term' be the common names or classes (as in the Appendix)?	According to the AOP wiki, the "Term" can be, latin name, common name or broader taxonomic group. Also, since

		these are structural terms which are chosen from a dropdown menu, it is not possible to change the "Term" manually without requesting a completely new term in the wiki.
Key Event Description (Event #1523)	Include a coma after 'shedding' This information on the ecdysis motor program should be added to the manuscript.	Included a comma after shedding. The ecdysis motor program is now also introduced in the introduction of the manuscript.
How it is Measured or Detected (Event #1523)	GIcNAc should be define	Done.

Reviewer # 3:

Section	**Comment**	**Answer**
How it is Measured or Detected (Event #1522)	This assay for CHS-1 activity actually seems more like an assay for chitin content. Is it suggested that this assay would be used to measure the formation of chitin when precursors are added to an enzyme extract? If this is the case, it should be made clearer. It does seem like this ELISA could also be considered as a method of detection for the KE "chitin cuticular chitin content."	Originally, the assay was developed to measure CHS activity using crude enzyme extracts (specified this in the text). But we agree that it can also be used to measure chitin content by using homogenates of chitin containing biological material (added this assay with specification in the KE page for "decrease, cuticular chitin content").
Key Event Description (Event #1523)	I agree that the description of ecdysis should be added to the manuscript. However, this paragraph might be better placed in the description of "premature molting."	Thanks, moved the description to the KE Page of "premature molting"
How it is Measured or Detected (Event #1523)	Can the ELISA method described on p. 10 be used here as well?	Yes, included this here.
Key Event Description (Event #1524)	As in the manuscript, this KE description needs to be better described and developed. How are these effects observed?	Added more description here and in the manuscript.
How it is Measured or Detected (Event #1524)	More examples and citations are needed in this section.	Done.