Systematic Evidence Map Protocol for Measurement Methods of Estrogenic, Androgenic, and Thyroid Hormones in Aquatic and Terrestrial Wildlife Species

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Introduction

Regulatory rationale

Within the EU, the approval of pesticides is regulated by Regulation (EC) No 1107/2009. An amendment to this Regulation (Regulation (EU) No 2018/605), which establishes the scientific criteria for the identification of pesticide active substances with endocrine disrupting (ED) properties, was published in April 2018. In June 2018, European Food Safety Authority, the European Chemicals Agency (ECHA) and the Joint Research Centre (JRC) published a guidance document on the implementation of the scientific criteria, this publication primarily addresses the Estrogen, Androgen, Steroidogenesis and Thyroid, known as 'EATS', modalities in vertebrates. It specifically included recommendations on how to perform hormonal measurements in mammalians. However, when it comes to non-mammalian vertebrate test guidelines (TG), there are a number of 'gaps' which need to be filled to fully address assessment of 'EATS' modalities and mirror mammalian TGs. For example, in the OECD test models which do address endocrine specific endpoints (i.e. amphibians and fish) there are no specific recommendations on which hormones to measure, how or when to measure hormones, or how to interpret the results. To harmonise vertebrate OECD TGs, these additional endpoints need to be investigated and therefore some guidance on how to perform, report and evaluate hormonal measurements in fish, birds and amphibians needs to be prepared.

Aim and objectives

The overall aim of this systematic evidence map is to collect and analyse data in support of the ED assessment (Estrogen, Androgen, Steroidogenesis and Thyroid) for non-target vertebrates in order to develop recommendations on how to perform, report and interpret hormonal measurements in fish, amphibians and birds in toxicity studies.

To support this aim, the specific objective of this evidence map is to collate literature that addresses the development, optimisation and/or validation of hormone measurement methods in fish, amphibian and bird species.

Methods

This protocol has been drafted with specific regards to the PRISMA-P (Preferred Reporting Items for Systematic review and Meta-Analysis Protocols) 2015 checklist[1] and is giving due consideration to the Code of Practice for the Conduct of Systematic Reviews in Toxicology and Environmental Health Research (COSTER)[2]. A preliminary draft was published on Zenodo on 10th May 2019 (doi: 10.2903/j.efsa.2018.5311) and comments were invited between 13th to 20th May. Responses collated via a google form (Appendix C) can be found in Appendix D. The protocol was revised in response to these comments and responses to individual comments can be found in Appendix E.

Eligibility criteria

Early decisions made during protocol development have significant impact on the scope and form of the systematic map. Protocol development is underpinned by imparting a common understanding of the context and motivation for the review. Well-formulated statements have a critical impact on other components of the review – including the literature search strategy, data extraction, synthesis and presentation of findings.

Question formulation follows a similar procedure as that for systematic reviews, i.e. using PI/ECO (population, intervention/exposure, comparator, outcome), PIT (population, index test, target condition) or PO (population, outcome) statements. For this project, research questions are related to the reliability and relevance of test methods for detection or diagnosis, in which case the population (P), index test (I) and target condition (T) must be specified. The initial PIT statement for is illustrated in Table 1.

Table 1. PIT statement

How do available methods to measure hormones related to the estrogen, androgen, steroidogenesis and thyroid hormones pathways in non-mammalian vertebrate species (birds, fish and amphibians) perform?	
Population	Species of fish, amphibians and birds
Index test	Invasive or non-invasive hormonal measurement methods (related to EATS pathways)
Target conditions	Detection of or supporting evidence for an endocrine disruption mode-of-action or adverse effect.

This PIT statement is operationalised as inclusion and exclusion criteria as described in Table 2 overleaf.

[1] Shamseer L, Moher D, Clarke M, Ghersi D, Liberati A, Petticrew M et al. (2015) Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015: elaboration and explanation *BMJ* ; 349 :g7647

[2] Whaley et al. "A Code of Practice for Conduct of Systematic Reviews in Toxicology and Environmental Health Research (COSTER)" Under development

Table 2. Eligibility criteria

		Inclusion criteria	Exclusion criteria
Population	Species of fish, amphibians and birds	Both wild and standard test species of fish, birds or amphibians, *in vivo* and *in vitro*	All other species that are not classified as fish, birds or amphibians
Index test	Invasive or non-invasive hormonal measurement methods (related to EATS pathways)	All sampling matrices including faeces or tankwater. Hormones related to the EATS pathways in fish, birds and amphibians. Estrogens, androgens and thyroid hormones are the main target of these searches but retrieved studies investigating CRH, cholesterol, FSH and/or LH will not be excluded.	Hormones not related to EATS pathways in fish, birds and amphibians, e.g. adrenaline and noradrenaline, stanniocalcin. Hormone dependent biomarkers such as vitellogenin.
Target conditions	Detection of or supporting evidence for an endocrine disruption mode-of-action or adverse effect.	Studies addressing methodological aspects of hormone measurements, e.g. method development, sample preparation and storage, method validation	Studies where pre-existing method(s) in the taxonomic group under consideration has been applied to measure hormones[3]

Information sources

Searches for peer-reviewed articles will be conducted in the following bibliographic databases:

- PubMed
- Web of Science All databases (selecting BioSIS Citation Index and SciELO in addition to the Core collection in the drop-down menu)
- MEDLINE
- Scopus
- Full text databases such as ScienceDirect

Further, in order to identify ongoing research, we will examine conference papers via resources like the British Library service **Zetoc** (http://zetoc.jisc.ac.uk/).

To identify grey literature not listed in databases, manual searches will be carried out using topic focused search engines such as **Mednar** (http://mednar.com/mednar/desktop/en/search.html) and **Environar** (https://environar.com/environar/desktop/en/search.html). Additional searches will be

[3] As part of data analysis, we will investigate how selected methods of particular interest have been applied using citation searches.

carried out in open access bibliographical databases such as **OpenGrey** (http://www.opengrey.eu/) which searches grey literature across Europe by interrogating open access items in institutional repositories.

Manual searches of the bibliography and citations of eligible studies will be carried out.

Finally, this can be complemented by targeted manual searches of open repositories on the website of European institutions such as EChA, EFSA, JRC, American institutions such as National Institute of Environmental Health Sciences (NIEHS), National Institute of Health (NIH) or the Food and Drug Administration (FDA) and, European national institutions including Bundesinstitut für Risikobewertung (BfR), Swedish Chemical Agency (KEMI), the Dutch National Institute for Public Health and the Environment (RIVM), Umwelt BundesAmt (UBA), the French National Institute for Industrial Environment and Risks (Ineris), the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) as well as those of interest groups such as the industry funded European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) or European Chemical Industry Council (CEFIC).

Language capabilities within the project team include German, Portuguese, Italian and French in addition to English. For studies in languages not covered by the project team and for which no English version can be located, the full text will be interrogated through the use of online tools such as Google Translate. It is not proposed to limit the timespan of literature searches.

Search strategy

Constructing search strategies for complex review questions can be challenging and has led to the use of multi-stranded strategies instead of a single combination of key elements, e.g. such as PI or IT. An initial series of four groups of search terms targeting either species, sampling matrix, hormones or method elements has been developed to be run sequentially and then combined using the Boolean operators 'AND' and 'OR'[4]. The result of pilot searches listing a total of 93 search terms and their combination in the three literature databases Web of Science, Scopus and Pubmed can be found in Appendix A.

The search strategy will be revised after review by EFSA and public invitation for comments. The quality of the revised literature search strategy will be assessed by testing for its ability to retrieve pre-identified key papers and by using the EFSA critical appraisal tool (CAT) for systematic/extensive literature searches[5]. The CAT focuses on both the search strategy; and the completeness of the information sources used.

Data management

Literature and all systematic review processes will be managed and coordinated with the support of the freely available online tool CADIMA established in a close collaboration between the Julius Kühn-Institut and the Collaboration for Environmental Evidence (https://www.cadima.info/index.php/area/evidenceSynthesisDatabase).

Relevance screening

The list of eligibility criteria will be applied to the merged reference list in duplicate, i.e. by two people working independently, and in two stages. In the first stage, only titles and abstracts will be checked for relevance to the study question. Clearly irrelevant studies will be excluded. The full text

[4] EFSA (2017). EFSA supporting publications 2017:EN-1207. 48 pp. doi:10.2903/sp.efsa.2017.EN-1207
[5] EFSA (2015) EFSA supporting publication 2015:EN-836. 65 pp. Appendix D.

of the resulting list of included references after title/abstract screening will then be examined for inclusion. The reason for exclusion of studies after assessment of the full text will be recorded.

Multiple reports of the same research (e.g. multiple publications, conference abstracts etc.) will not be excluded but instead the methodological information from each of the reports shall be collated as part of the data extraction process as one unit of evidence.

The CADIMA online tool facilitates the process of consistency check by identifying disagreement between the two evaluators. Disputes will be arbitrated and resolved by a third party, the project manager and most senior ecotoxicology expert. For quality control purposes, the percentage agreement between the two independent evaluators and kappa statistic will be reported.

The inter-rater reliability (level of agreement between two or more assessors) of clearly formulated inclusion and exclusion criteria will be also piloted and eligibility criteria shall be clarified and amended accordingly, if necessary.

Data extraction

As this project is concerned with the validation of methods for hormonal measurements, data related to the critical appraisal of methods is central to this exercise and forms part of the data extraction and coding itself. There is therefore an overlap between data extraction and risk-of-bias. Furthermore, the latter step is not automatically required in systematic evidence mapping. In this specific instance, it was found that this would duplicate data extraction and risk-of-bias was therefore not included. An initial data extraction template has been created comprising of elements relevant for different types of studies, e.g.:

- meta-data (authors, date, journal name or report number)
- information about the test system, specifically:
    - Species name(s)
    - Taxonomic group (fish, bird or amphibian)
    - Life stage and gender of the organisms
    - Name of the specific bioassay, if relevant
- information about the study design, including:
    - whether it investigated a specific intervention or exposure, and if so details of any chemical exposure(s)
    - Hormones measured and associated endocrine modality(ies)
- Details of the sampling for hormone measurements, particularly:
    - Whether the sampling method was invasive
    - The matrix sampled
    - The amount (e.g. volume or weight) of sample required for analysis
    - Whether samples needed to be pooled across several individual
- Details of the hormone measurement method including;
    - The type of method and method name
    - Whether a standard operating procedure exists (SOP)
    - The use of internal standards
    - Sensitivity (limit of detection and/or limit of quantification)
    - Any reported information relevant to specificity

At this stage, the aim of this data extraction template is to capture essential information that can be used to categorise different methods for further data analysis rather than attempt to capture every

relevant detail reported in each article. This is achieved by the use of drop-down menus restricting possible answers to lists of categories whenever possible.

This template was piloted by extracting data from four representative articles by four evaluators in parallel duplicates. As a result of this exercise, the data extraction template was amended and corrected. The following items were added;

- The aim of the hormonal measurements,
- Whether sample extraction step(s) was/were necessary and brief details about methods used,
- Brief information about the accuracy and precision of the method.

The amended data extraction temple can be found in Appendix B. This amended version of the data extraction template will be further revised following review by EFSA colleagues and public invitation for comments. The revised version will again be piloted with a different set of relevant studies and a set of guidelines for evaluators as to what is considered a 'unit of evidence', or how information is collated when an article reports information about different species, methods, hormones etc.

Data analysis

A description of the volume and characteristics of the evidence base collated in the previous step, such as the availability of analytical methods for different hormones and taxonomic groups and if data permits indicators of performance (e.g. accuracy, precision, sensitivity, selectivity) of these different methods will be visually summarised and presented using the capabilities of the data visualisation software Tableau.

An in-depth qualitative analysis will also be carried out by combining results by taxonomic group, method type and endocrine modality or hormone measured. This may require additional searches to identify the papers that have cited an article about a given method to gauge how widely and reliably it has been applied in the context of chemical testing.

This analysis will guide the design of a questionnaire addressed to laboratories that routinely carry out regulatory ecotoxicity studies. This questionnaire will seek opinions on technical and scientific aspects as well practical feasibility aspects that are rarely addressed in the scientific literature such as availability of equipment, technical expertise required and costs involved.