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Metabolism-based category formation for the prioritisation of genotoxicity hazard assessment for plant protection product residues (part 3): Strobilurins

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ABSTRACT

In dietary risk assessment of plant protection products, residues of active ingredients and their metabolites need to be evaluated for their genotoxic potential. The European Food Safety Authority recommend a tiered approach focussing assessment and testing on classes of similar chemicals. To characterise similarity, in terms of metabolism, a metabolic similarity profiling scheme has been developed from an analysis of 46 chemicals of strobilurin fungicides and their metabolites for which either Ames, chromosomal aberration or micronucleus test results are publicly available. This profiling scheme consists of a set of ten sub-structures, each linked to a key metabolic transformation present in the strobilurin metabolic space. This metabolic similarity profiling scheme was combined with covalent chemistry profiling and physico-chemistry properties to develop chemical categories suitable for chemical prioritisation via read-across. The method is a robust and reproducible approach to such read-across predictions, with the potential to reduce unnecessary testing. The key challenge in the approach was identified as being the need for metabolism data and individual groups of plant protection products as the basis for the development of such profiling schemes.

1. Introduction

The European Food Safety Authority (EFSA) requires an assessment of the genotoxicity potential for fungicide residues, where the term residue is defined as any compound associated with the active ingredient (EFSA (Scientifc Committee), 2016). More specifically, residues that humans could potentially be exposed to through their diet need to be assessed for hazard, including genotoxicity. To this end, EFSA have published guidance detailing a workflow that includes category formation and read-across for the prediction of genotoxicity (EFSA (Scientific Committee), 2011). Category formation relates to deriving criteria describing chemical similarity and demonstrating adherence to those criteria for a set of chemicals, while read-across relates to predicting toxicological data-gaps utilising existing data from a chemical (or chemicals) within the category (Schultz et al., 2015).

The general approach is that plant protection residues should not increase the hazard to humans (and livestock). Thus, within a set of (structurally similar) plant protection residues, a category, a representative number need to have *in vitro* and/or *in vivo* data for gene mutation as well as structural and numerical chromosomal aberration. For a negative read-across prediction, data (for the category members) from the Ames test (gene mutation) and an *in vitro* micronucleus test (structural and numerical chromosomal aberration) are the minimal requirement to enable the data gap to filled. The availability of additional negative *in vivo* data would add further weight of evidence to the read-across prediction (especially where exposure to the bone marrow has been demonstrated from toxicokinetic studies). If a read-across prediction of genotoxicity is negative, then no further experimental testing is required under the EFSA guidance (EFSA (Scientific Committee), 2011). In contrast, a positive read-across prediction for

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genotoxicity requires further experimental data to be generated in a tiered approach. For example, if an initial *in vitro* micronucleus test confirms the positive read-across prediction for chromosome damage, an *in vivo* micronucleus test would be triggered.

The key step in the use of the category formation approach is the ability to confidently define 'similarity' between chemicals (Enoch et al., 2010; Enoch et al., 2013; OECD, 2007). In terms of the use of category formation in the EFSA genotoxicity workflow noted above, defining similarity is relatively straightforward for potentially genotoxic chemicals. This is due to the key molecular initiating event for DNA-reactive genotoxicity being the formation of a covalent bond between nucleophilic centres in DNA and a chemical capable of behaving as an electrophile (either directly or after metabolic activation) (Enoch and Cronin, 2010, 2012; Benigni and Bossa, 2008; Benigni et al., 2009; Mekenyan et al., 2004, 2007; Serafimova et al., 2007). The associated chemistry can be encoded easily as structural alert-based *in silico* profilers that enable chemicals to be assigned to a category based on the presence of a common alert. In contrast, defining similarity between chemicals that lack an alert for DNA reactivity is more challenging due to the lack of such key structural features (Schultz et al., 2018).

Recent research has shown that "structural space alerts" can be defined from an analysis of the genotoxicity and metabolism data available in the Draft Assessment Report/Renewal Assessment Report (DAR/RAR) documents of plant protection products (available from the EFSA website) (Enoch et al., 2022a, 2022b). This analysis showed how metabolic information could be used to drive the development of the structural space alerts – enabling chemical groupings to be defined in which common metabolic pathways were present in the analogues – something that has been identified as being a key measure of similarity (Gadaleta et al., 2020; Yordanova et al., 2021; Boyce et al., 2022). The analysis also showed how these structural space alerts could be used in conjunction with other profiling schemes (for example, those available in the OECD QSAR Toolbox) to build a weight of evidence for the prediction of Ames, chromosomal aberration, and the micronucleus assays via read-across, with predictions being possible for both *in vitro* and *in vivo* endpoints. However, the key limitation with the previously published structural space alerts was that each alert was not explicitly linked to a single metabolic transformation. Thus, the aim of the current study was to expand the previously published structural space alert approach into a metabolic profiling scheme in which such information is included. The idea being to make the resulting read-across predictions more transparent from a metabolism point of view. The approach is exemplified using the strobilurin group of plant protection products.

2. Method

2.1. Dataset

A dataset of 46 strobilurin fungicide active ingredients and metabolites with either Ames, *in vitro* chromosomal aberration or *in vivo* micronucleus test results were extracted from the 10 publicly available DAR/RAR documents (available from efsa.europe.eu). Genotoxicity data were extracted for all compounds that had been directly tested or for those chemicals that satisfied the definition of a major metabolite (in the rat) as outlined in the EFSA guidance (EFSA (Scientifc Committee), 2016). In all cases bioavailability had been proven for the *in vivo* test data (for example, exposure to the bone marrow in the micronucleus test). The dataset, termed the 'strobilurin genotoxicity dataset' contained the following test results (*in vitro* assays with S9 fraction, Ames tests in the standard battery).

- Ames: 46 chemicals (all negative)
- *In vitro* chromosomal aberration: 30 chemicals (23 negative, 7 positive)
- *In vivo* chromosomal aberration: 1 chemical (negative)
- *In vitro* micronucleus: 14 chemicals (12 negative, 2 positive)
- *In vivo* micronucleus: 24 chemicals (all negative)

All chemical structures and associated genotoxicity data are available in the Supplementary Information.

2.2. Metabolic similarity profiling scheme

The development of the metabolic similarity profiling scheme utilised the following two steps.

1. Definition of the metabolic maps for the strobilurin fungicides: This analysis involved inspection of the available metabolism data in the 10 DAR/RAR documents to identify metabolic transformations common to this class of fungicides. These metabolic transformations were the oxidation, reduction, and hydrolysis reactions centred on either the methoxy-acrylate, oximino-acetamides, oximino-acetates, dihydro-dioxazines, methoxy-acetamides, and methoxy-carbamates moieties.
2. Sub-structure identification: Common sub-structures were then identified from the metabolic maps developed in step 1. These sub-structures defined the key functional groups present within chemicals identified as undergoing the key transformations defined in the metabolic maps.

2.3. Chemical profiling

Chemicals in both datasets were profiled using the profiling schemes within the OECD QSAR Toolbox (V4.1.1). A subset of the available profilers was utilised based on the results of a previous study into their suitability for read-across predictions within the plant protection chemical space (Enoch et al., 2022a, 2022b). These profilers were (CA is chromosomal aberration and MNT is the micronucleus test):

- DNA alerts for AMES, CA and MNT by OASIS
- Protein binding alerts for CA by OASIS

3. Results and discussion

The aim of this study was to develop a metabolic similarity profiling scheme to enable the genotoxicity of the strobilurin group of plant protection products to be predicted via read-across. A series of substructures linked to key metabolic transformations were defined based on an expert analysis of the metabolic information available in the DAR/RAR documents for the strobilurin group of plant protection products. The key advantage of the approach being that the resulting category members undergo a common set of metabolic transformations, which increases the robustness, reliability, and repeatability of the approach.

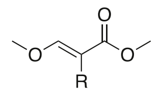
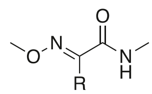
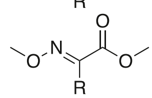
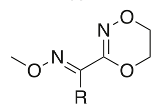
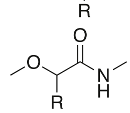
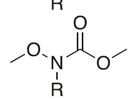
3.1. Available metabolism data

Metabolism data were collected from the eight publicly available DAR/RAR reports for the strobilurin fungicides. These active ingredients are divided into six classes based on the strobilurin functional group (www.frac.info) as shown in Table 1 (methoxy-acrylates, oximino-acetamides, oximino-acetates, dihydro-dioxazines, methoxy-acetamides, and methoxy-carbamates). Inspection of the available DAR/RAR documentation for these compounds showed the primary site of metabolism to be the strobilurin functional group (functional groups shown in Table 1). In addition, the DAR/RAR documents also showed metabolic cleavage of the ether linkage present in the two scaffolds to be common across this class of fungicides (Fig. 1).

3.2. Metabolic map development

Given that the majority of the metabolism for the strobilurin fungicides occurs at the strobilurin functional group, it was decided that these

Table 1
Definition of the strobilurin chemical classes for which metabolism data are publicly available publicly (in all cases R = aromatic ring, FRAC = Fungicide Resistance Action Committee).

Active ingredient	FRAC class	Strobilurin functional group
Picoxystrobin Azoxystrobin	Methoxy-acrylates	
Dimoxystrobin	Oximino-acetamides	
Kresoxim-methyl Trifloxystrobin	Oximino-acetates	
Fluoxastrobin	Dihydro-dioxazines	
Mandestrobin	Methoxy-acetamides	
Pyraclostrobin	Methoxy-carbamates	

transformations would be used to define metabolic similarity within a chemical category. An analysis of the metabolic information available in the DAR/RAR documents for the rat resulted in the creation of six metabolic maps, one for each class of strobilurin fungicide. Fig. 2 demonstrates an example of such a map for the methoxy-acrylate strobilurins which shows the parent structure to potentially undergo five key metabolic pathways:

A. Dealkylation: this reaction involves the oxidation of the methoxy enol ether group to an alcohol. Inspection of the available data did not show an equivalent transformation for simple aliphatic methoxy groups (i.e., those lacking the adjacent alkene moiety).
B. Ester hydrolysis: this reaction involves hydrolysis of the ester group, to give the corresponding carboxylic acid. This type of transformation was extremely common in the metabolic maps in the DAR/RAR documents, with most metabolites that contained an ester undergoing hydrolysis.
C. Alkene reduction: this reaction involves the reduction of the alkene moiety to an alkane. This transformation was noted as a key step in the overall metabolism of the methoxy-acrylate moiety as it enables two further transformation types to occur on the resulting alkane (pathways D and E).
D. Alcohol oxidation: the presence of a primary alcohol moiety enables a two-step oxidation reaction to occur in which the alcohol is converted to an aldehyde and then a carboxylic acid. The last step in this pathway involves the cleavage of the carboxylic acid moity – this only occurs for aliphatic carboxylic acids (i.e., those connected to a primary carbon atom).
E. Hydroxylation: the presence of a secondary or tertiary carbon atoms between an aromatic ring and carbonyl moiety enables a hydroxylation reaction to occur. This is a widespread transformation that occurs in most cases featuring this structural feature according to the data in DAR/RAR documents.

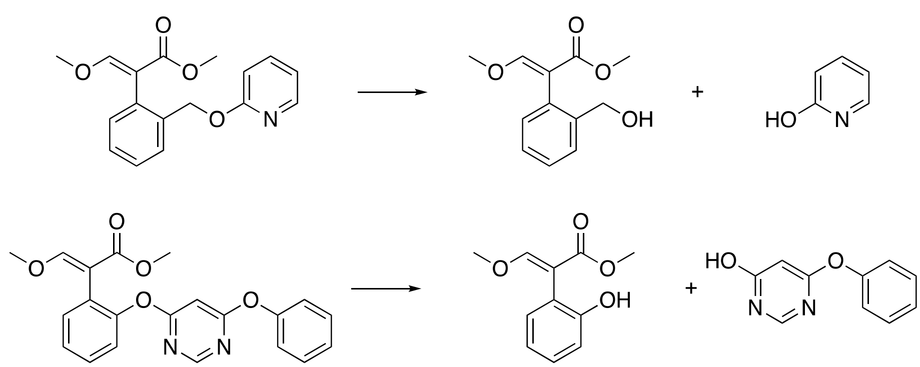


Fig. 1. Metabolic cleavage of the ether linkages in the two scaffolds present in the active ingredients listed in Table 1 (methoxy-acrylate strobilurin functional group shown).

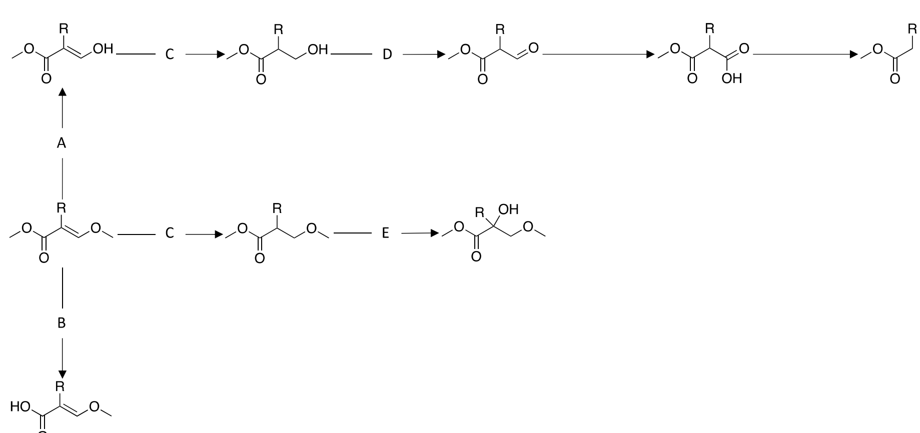


Fig. 2. Metabolic map for the methoxy-acrylate strobilurin fungicides from an analysis of the DAR/RAR documents for picoxystrobin and azoxystrobin (R = aromatic ring, transformations defined from an analysis of rat metabolism).

It is important to realise that the metabolic maps represent a summarised overview of the key common transformations for each strobilurin group. The metabolic information in the DAR/RAR documents is significantly more complex than (for example) the map shown in Fig. 2. For example, ester hydrolysis is only shown once in Fig. 2, even though the available metabolic data indicated that most esters undergo hydrolysis. The same is true of the other metabolic reactions (in that if the relevant structural features are present in a molecule, then they are likely to occur). The metabolic maps for the other strobilurin groups are available in the Supplementary Information.

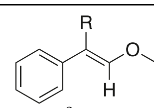
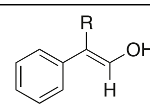
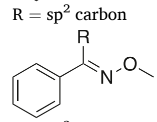
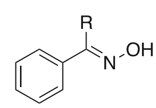
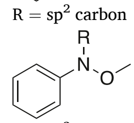
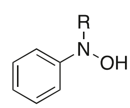
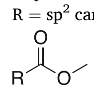
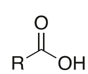
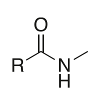
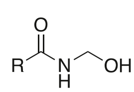
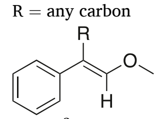
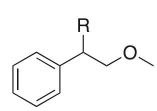
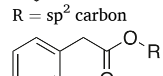
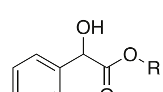
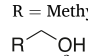
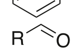
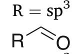
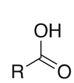
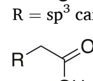
3.3. Metabolic similarity profiling scheme

The initial step in the development of the metabolic similarity profiling scheme was the systematic naming of the transformations. This was important as the similarity within a category was defined based on the presence of a set of common metabolic transformations shared by the target chemical (the chemical with a genotoxicity data gap) and the analogues (see case study for further details). Thus, the transformations shown in the metabolic map in Fig. 2 were named as detailed in Table 2. The naming system being based on the most common phase 1 transformations as defined in references (21, 22). Two levels of naming were employed, these being:

1) Metabolism class. This defines the generic type of metabolic reaction occurring between two metabolically related structures in the map in terms of oxidation, reduction, or hydrolysis.
2) Metabolic transformation: This defines the type of transformation occurring at a functional group level that is required to convert one structure into another.

A similar analysis was performed for the other classes of strobilurin fungicides enabling a set of ten common transformations to be defined as shown in Table 2 (the metabolic maps for the remaining strobilurin fungicides are available in the Supplementary Information). Within Table 2 the parent sub-structure defines the structural space alert used to profile the dataset to identify compounds with genotoxicity data from the dataset. The analysis showed four of the seven sub-structures to be present in more than one class of strobilurin fungicide, with the remaining six being present only in the methoxy-acrylates class. In addition, most sub-structures have multiple genotoxicity data associated with them (note, that more than one sub-structure can be present in each molecule – in such cases the associated genotoxicity data shown in Table 2 has been counted against each sub-structure present). The

Table 2
Common metabolic transformations, and associated genotoxicity data, defined for the strobilurin group of plant protection products (where CA = chromosomal aberration, MNT = micronucleus test, N/A = non available, '-ve' indicates a negative result, '+ve' indicates a positive result – the preceding numbers indicating how many chemicals that were negative or positive, all positive *in vitro* CA data were subsequently shown to be negative in an *in vivo* MNT).

ID	Metabolism Class	Metabolic Transformation	Sub-structures		Strobilurin	Genotoxicity data
			Parent	Child		
1	Oxidation	Dealkylation	 R = sp^2 carbon		Methoxy acrylates	Ames: 3 -ve *In vitro* CA: 1 +ve; 1 -ve *In vivo* CA: 1 -ve *In vivo* MNT: 2 -ve
2	Oxidation	Dealkylation	 R = sp^2 carbon		Oximino acetamides Oximino acetates Dihydro dioxazines	Ames: 22 -ve *In vitro* CA: 12 -ve; 1 +ve *In vitro* MNT: 7 -ve *In vivo* MNT: 10 -ve
3	Oxidation	Dealkylation	 R = sp^2 carbon		Methoxy acetamides Methoxy carbamates	Ames: 4 -ve *In vitro* CA: 3 -ve; 1 +ve *In vitro* MNT: 1 -ve; 1 +ve *In vivo* MNT: 4 -ve
4	Oxidation	Ester hydrolysis	 R = any carbon		Methoxy acrylates Oximino acetamides Oximino acetates	Ames: 14 -ve *In vitro* CA: 9 -ve, 2 +ve *In vitro* MNT: 6 -ve; 2 +ve *In vivo* CA: 1 -ve *In vivo* MNT: 8 -ve
5	Oxidation	Aliphatic hydroxylation	 R = any carbon		Methoxy acetamides Oximino acetamides	Ames: 13 -ve *In vitro* CA: 8 -ve, 1 +ve *In vitro* MNT: 3 -ve *In vivo* MNT: 7 -ve
6	Reduction	Alkene reduction	 R = sp^2 carbon		Methoxy acrylates	Ames: 3 -ve *In vitro* CA: 1 -ve, 1 +ve *In vivo* CA: 1 -ve *In vivo* MNT: 2 -ve
7	Oxidation	Benzylic hydroxylation	 R = Methyl, hydrogen		Methoxy acrylates	Ames: 1 -ve
8	Oxidation	Alcohol oxidation	 R = sp^3 carbon		Methoxy acrylates	No data
9	Oxidation	Aldehyde oxidation	 R = sp^3 carbon		Methoxy acrylates	No data
10	Reduction	Carboxylic acid cleavage	 Any carbon		Methoxy acrylates	No data

exceptions were sub-structure seven which only has a single Ames test and sub-structures eight, nine and ten that have no data associated with them.

3.4. Case study: data gap filling for metabolite R234886 from azoxystrobin

The metabolic information outlined in Table 2 can be utilised to identify 'metabolically similar' compounds to fill data-gaps via read-across. As an example, consider the metabolite R234886 of the active ingredient azoxystrobin. Inspection of the available data for this metabolite showed only a negative Ames study, meaning data gaps existing for structural and numerical chromosomal aberration. Metabolic profiling of this chemical shows that it to potentially undergo two metabolic transformations: dealkylation of the methoxy group (sub-structure 1 in Table 2) and alkene reduction (sub-structure 6 in Table 2). Inspection of the available data showed there to be two chemicals in the dataset that contained both sub-structures. However, both analogues also contained an additional sub-structure related to ester hydrolysis (sub-structure 4 in Table 2) that was not present in the target chemical. However, the available data associated with this type of transformation are negative in the related genotoxicity assays (Table 2). The chemical structure and associated genotoxicity data for the metabolically similar analogues identified for the R234886 are shown in Table 3.

An advantage of the proposed approach is the ability to identify additional analogues from the other strobilurin chemical classes that have the same metabolic profile as the target chemical as sources of secondary evidence. Inspection of Table 2 shows there to be a further two sub-structures associated with corresponding dealkylation event present in the other five classes of strobilurin plant protection products (sub-structures 2 and 3 in Table 2). These data add a significant weight of evidence that the dealkylation metabolic pathway does not lead to genotoxicity – especially the negative Ames and *in vivo* micronucleus test results. Finally, none of the compounds triggered any structural alerts within the OECD QSAR Toolbox when profiled with the OASIS endpoint specific profilers for genotoxicity. The sole use of the OASIS profilers being supported by previous studies in which the applicability domains of the various genotoxicity profiling schemes within the OECD QSAR Toolbox was assessed (Enoch et al., 2022a, 2022b). Taking all the available data together enabled prediction of R234886 as negative for structural and numerical chromosomal aberration via read-across. Given that the bioavailability of the test compounds had been previously demonstrated for all the *in vivo* test results (an important additional piece of evidence in the absence of *in vitro* chromosomal aberration or micronucleus test data), no further experimental testing would be required within the EFSA guidance (EFSA (Scientific Committee), 2011).

3.5. Metabolic similarity workflow

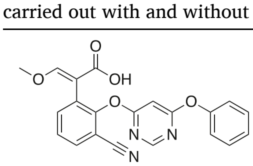
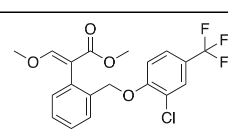
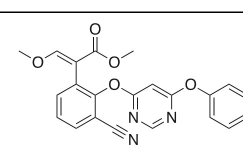
The key aim of this study was to develop a metabolic similarity profiling scheme for the strobilurin group of plant protection products. It is important to outline how the approach outlined in this study could be applied to other class of plant protection products, given the availability of metabolic information in a set of DAR/RAR documents. This is as outlined in step 1–6 below. As with the previous work in this area (Enoch et al., 2022a, 2022b), the protocol also enables other lines of evidence to be included, for example, predictions from endpoint specific *in silico* profiling schemes. In principle this approach can be applied to any dataset using the workflow outlined as follows:

1. Key functional group/groups identification: the initial step in the analysis is the identification of a key functional group of metabolic concern within the agrochemical class of interest. Typically, this will be a single moiety of high concern and/or the site of primary metabolism within a class of agrochemicals. The method is less applicable to classes of agrochemicals that undergo complex metabolism involving multiple functional groups.
2. Metabolic map development: having identified the key functional group (or groups) of metabolic concern, the next step is to gather available metabolic data to enable the known transformations of this group/these groups to be defined. The most common source of these types of data are the publicly available DAR/RAR documents.
3. Transformation naming: this is the key step in the approach in which expert judgement is used to name the transformations that have been identified during the metabolic map development. The naming convention needs to be systematic as the transformation names are used to define metabolic similarity within a category. In the current study several key references sources have been identified to aid this process (Testa et al., 2007; Trager et al., 2007).
4. Sub-structure definition: sub-structures that define the key functional groups capable of undergoing the metabolic transformations identified in step 3 are defined. These sub-structures enable analogues to be identified based on the presence of a common metabolic transformation present in the target and analogues.
5. Identification of metabolically similar analogues: the sub-structures associated with the key transformations identified in step 4 are utilised to identify analogues. All possible analogues are returned in situations where a target chemical has more than one sub-structure present.
6. Additional profiling: evidence from other *in silico* profiling schemes such as the endpoint specific profilers in the OECD QSAR Toolbox can be added to further substantiate the weight of evidence. For a recommendation of suitable profiling schemes see references (Enoch et al., 2022a, 2022b).

4. Conclusions

This study has outlined the development of a metabolic similarity profiling scheme for the strobilurin group of plant protection products to enable genotoxicity data-gaps to be filled via read-across. In addition, a protocol has been developed that enables such profiling schemes to be developed for any class of plant protection products for which metabolism information is available in DAR/RAR documents. The key

Table 3
Metabolic profiling results for metabolite R234886 corresponding to dealkylation of the methoxy group and the reduction of an alkene (sub-structures 1 and 6 in Table 2). Where CA = chromosomal aberration, MNT = micronucleus test, R/A = read-across prediction. All *in vitro* data carried out with and without S9 liver fractions.

		
R234886 (Target) Ames: negative Prediction: *In vivo* CA, MNT: negative (R/A)	Picoxystrobin Ames, *In vitro* CA, *In vivo* MNT: negative	Azoxystrobin Ames, *In vivo* CA, *In vivo* MNT: negative *In vitro* CA: positive

advantage to the approach being that all the resulting analogues identified within a category are metabolically related to the target chemical of interest. However, the proposed workflow does rely on the availability of metabolic data (typically from DAR/RAR documents) and expert judgement around the key site (or sites) of metabolism upon which to focus the development of the metabolic maps. In addition, it is possible that metabolites may fall out of the scope of the defined structural space alerts. The approach was exemplified using a case study approach that showed these chemical categories could be used to predict genotoxicity or prioritise strobilurin residues for further targeted testing. Importantly, this approach enabled an assessment of gene mutation as well as structural and numerical chromosomal aberrations. Finally, the approach presented enables multiple lines of evidence to be included to strengthen the weight of evidence of the read-across prediction.

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CRediT authorship contribution statement

S.J. Enoch: Conceptualization, Writing – original draft, Writing – review & editing. **Z. Hasarova:** Formal analysis, Data curation. **M.T.D. Cronin:** Funding acquisition, Writing – review & editing. **K. Bridgwood:** Writing – review & editing. **S. Rao:** Writing – review & editing. **F. M. Kluxen:** Writing – review & editing. **M. Frericks:** Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Zuzana Hasarova reports financial support was provided by Crop Life Europe.

Data availability

All data are available in the supplementary information

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yrtph.2023.105484.

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