



Metabolomics as read-across tool: A case study with phenoxy herbicides



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ABSTRACT

New technologies, such as metabolomics, can address chemical grouping and read across from a biological perspective. In a virtual case study, we selected MCPP as target substance and MCPA and 2,4-DP as source substances with the goal to waive a 90-day study with MCPP. In order to develop a convincing case to show how biological data can substantiate read across, we used metabolomics on blood samples from the 28-day studies to show the qualitative and quantitative similarity of the substances. The 28-day metabolome evaluation of source substances and the target substance indicate liver and kidneys as target organs. 2,4-DP was identified as the best source substance. Using the information of the 90-day 2,4-DP study, we predicted MCPP's toxicity profile at 2500 ppm: reduced food consumption and body weight gain, liver and kidney weight increases with clinical-pathology changes and a moderate red blood cell parameter reduction. NOEL prediction for MCPP was below that of 2,4-DP (<500 ppm), and similar to that of MCPA (≥ 150 ppm). Qualitatively, these predictions are comparable to the results of the real MCPP 90-day study in rats (reduced food consumption and body weight gain, weight increases and clinical-pathology changes in liver and kidneys, reduced red blood cells values). Quantitatively, the predicted NOAEL (150 ppm) is similar to the actual study (NOEL = 75 ppm, NOAEL ≤ 500 ppm). Thus, the 90-day rat toxicity study of MCPP could have been waived and substituted by the 90-day results of 2,4-DP by using metabolome data of 28 day studies.

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1. Introduction

The information requirements according to the REACH legislation, and the number of chemicals involved, lead to a very significant increase of animal testing. The REACH legislation, in principle notes that animal testing should be the last resort and promotes the development and use of alternative methods. However, with the exception of some less complex studies (e.g. skin and eye irritation), very little progress has been made to have validated and regulatory acceptable alternative methods in place for REACH testing (Hoefer et al., 2004; Hartung and Leist, 2008). Grouping of chemicals and subsequent read across from data rich chemicals belonging to the group is probably the most efficient way to provide the required safety information, while keeping the amount of animal testing to an absolute minimum. The big question here is the quality of the grouping process. Read-across entails the use of relevant

information from analogous substances (the 'source' information) to predict properties for the 'target' substance(s) under consideration. Grouping and read-across may be based purely on structural similarity, however, with some risk of error. It would therefore seem prudent to include and take into account some biological data in the grouping process, whenever possible (van Ravenzwaay et al., 2012). These may come from in vitro studies, or could be derived from a limited number of base set animal studies. Omics technologies could serve as an important tool to enhance the quality and quantity of data obtained during regulatory toxicity testing (ECETOC, 2008; ECETOC, 2010).

ECHA has the obligation to evaluate if submitted read-across cases are sufficiently convincing to substitute these for standard tests. In response to this challenge ECHA developed and published the Read-Across Assessment Framework (RAAF) http://echa.europa.eu/documents/10162/13628/raaf_en.pdf. In this framework read-across approaches are assessed through the use of different scenarios and the quality of the case is consistently evaluated based on a number of predefined criteria. If supporting evidence is provided for a read-across case then this may be taken into account

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when conducting an assessment according to the RAAF. Many new approaches and methodologies for investigating properties of chemicals have been developed over the past years. To assess the value of these new approach methodologies (NAMs), ECHA organized a workshop in Helsinki in April 2016 called “Topical Scientific Workshop on New Approach Methodologies in Regulatory Science”. The present paper was prepared as a case study for this workshop using metabolomics as a NAM to support read across.

In this paper we describe how metabolomics, can be used to address chemical grouping and read across from a biological perspective. The goal was to provide a convincing case to waive a 90-day rat study for the target substance MCPP (also referred to as Mecoprop or Mecoprop-p). Two other phenoxy-herbicides, MCPA and 2,4-DP (also referred to as Dichlorprop or Dichlorprop-P) were selected as source substances. It should be noted here that one of the requirements to serve as a case study was a substantial chemical similarity as specified in the RAAF. MCPP and 2,4-DP are phenoxypropionic acids and have a chiral centrum. In the past, these compounds were produced as racemic (50:50) mixtures of the two enantiomers. Since the 1990's the production has been modified to only produce one enantiomer (in documents generally specified by the addition of -p to the name of the compounds, e.g. mecoprop-p) which has the highest herbicidal activity. As the herbicidal activity is related to a plant specific receptor, not present in animals, the toxicity of racemic mixture and single enantiomer was shown to be identical. The modern toxicological package for both compounds has been generated in the 1990's and 2000's with the single enantiomer. The metabolome studies presented here were also performed with the single “-p” isomer. It should also be noted, that there are more phenoxy herbicides than the ones used for this case study, in particular 2,4-D, these, however were not included here, because of a lack of appropriate metabolomics data.

Within the context of ECHA's RAAF we work with the category approach, scenario 4 or 6. This scenario covers the category approach for which the hypothesis is based on different compounds, which have the same type of effect(s). For the REACH information requirement under consideration, the effects obtained in studies conducted with different source substances are used to predict the effects that would be observed in a study with the target substance if it were to be conducted. Concerning the strength of the effect (i.e. the major differences between cat 4 and 6) we would like the data to speak for itself and make a reasonable conclusion when all data are considered together. The overall purpose of this paper is to demonstrate the possibilities to assess toxicity by means of multi-parameter ‘omics sciences’, in this case particularly metabolomics.

For the read across case, the situation is as follows: there is an adequate 28-day rat study with MCPA, but only limited 28-day information for 2,4-DP. For all three substances, metabolome data from 28-day studies are available. The metabolome information is used for two purposes: 1) to predict the toxicological profile of each of the compounds, and 2) to compare the similarity of the metabolome of the source substances with the target substance and select the most appropriate one, to make a prediction of the 90-day toxicity in rats of the target compound. For both source substances 90-day studies are available. Finally, we compare the predicted outcome for the target substance with the real outcome.

1.1. Identity of the target substance

Structural information as well as phys-chem data on the target substance MCPP as well as on the source substances MCPA and 2,4-DP are depicted in Fig. 1.

The target substance and source substances are structurally similar. The target substance MCPP is a phenoxypropionic acid, and

as such comparable with phenoxypropionic acid 2,4-DP. The target substance has a methyl and chlorine substituent in the 2,4-position, and this part of the molecule is thus most similar with MCPA. The structural similarities of the compounds can be quantified by Tanimoto Scores (Fig. 2).

The different parameters of acute toxicity for the target substance and the source substances are listed in Table 1.

1.2. Conclusion

Acute Toxicity: the acute toxicity of the target substance and the source substances are comparable.

Mutagenicity: overall there are no concerns about the genotoxicity of the target and source compounds.

2. Absorption, distribution, metabolism & excretion

Absorption, distribution, metabolism and excretion, in short ADME, parameters are available for all three substances (MCPP was reviewed by California Environmental Protection Agency, 1999; MCPA was reviewed by JMPR, 2012; 2,4-DP was reviewed by California Environmental Protection Agency, 2002). The ADME results are summarized in Table 2. For all substances ^{14}C – phenyl labelled test substance was administered once by gavage as an aqueous CMC suspension to rats. Animals were maintained in metabolism cages. Studies were basically performed according to the respective OECD and US_EPA test guidelines for kinetics and metabolism.

Overall, bioavailability for target and source substances is high (>90% at low dose levels), to a somewhat lesser extent at higher dose levels. For all substances there is rapid elimination predominantly through the urine (low dose levels 80–90%) at high dose levels to a slightly lesser extent. Fecal elimination accounts for ca. 10% or less at low dose levels, and increases up to ca. 20% at high dose levels. Reduced urinary excretion and increased fecal excretion at high dose levels indicate a slightly reduced bioavailability at high dose levels. There is no elimination through the expired air. Fast elimination is reflected in relatively short, less than 8 h, and comparable half-lives. The unchanged parent compound is for all three substances by far the major component in the blood. Metabolism is limited to the production of one or a few minor metabolites (e.g. for MCPA: hydroxylation of the methyl group of the alcohol (HMCPA), followed by a second hydroxylation to form the acid (CCPA)), some of which have been tested for systemic toxicity and shown to be less toxic (van Ravenzwaay et al., 2005). There were no major differences between male and female animals. In conclusion, the ADME properties of the target and sources substances are substantially similar.

3. Twenty-eight-Day toxicity studies

There are only few 28-day toxicity studies available in the public literature and most of these studies have been performed at relatively low dose levels. Hence, the toxicological profiles following 28 days of compound administration are not very well defined (with the exception of MCPA). The findings of these studies have been summarized in Table 3.

3.1. MCPA (van Ravenzwaay et al., 2005)

Five male and five female Wistar rats received MCPA at a dietary concentration of 2000 ppm for four weeks, with examinations according to OECD guideline 407. Test substance intake was 166 and 172 mg/kg body weight/day for males and females respectively.

MCPA caused no clinical signs either during the study or in the

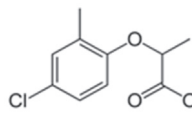
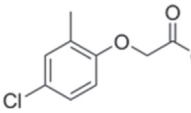
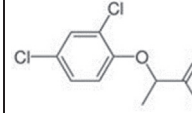
		Target Substance	Source Substances	
		MCPP	MCPA	2,4-DP
CAS number		93-65-2	94-74-6	120-36-5
Chemical name		(RS)-2-(4-Chloro-2-methylphenoxy)propanoic acid	2-methyl-(4-chlorophenoxy)acetic acid	(2R)-2-(2,4-dichlorophenoxy)propionic acid
Appearance		solid	solid	solid
Solubility in	water [mg/l] (20°C)	900	300	720
	ethanol [g/kg] (20 °C)	> 1000	> 1000	153
Linear Formula		C10H11ClO3	C10H9ClO3	C9H8Cl2O3
[°C]		94 to 95	115.4 to 116.8	116 to 120
Density [g/cm³]		1.28	1.56	1.4
LogP		3.13	3.25	3.43
Structural formula:				
Molecular Weight [g/mol]		214.65	200.62	235.06

Fig. 1. Chemical structure and some physico-chemical data for MCPP, 2,4-DP and MCPA.

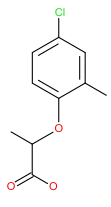
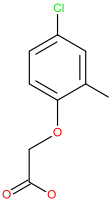
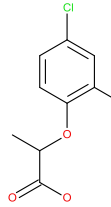
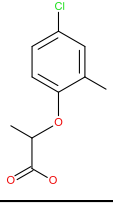
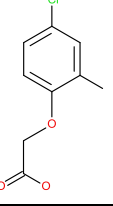
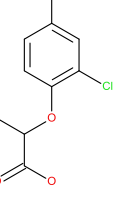
			
	MCPP	MCPA	2,4-DP
	MCPP	75.0%	96.0%
	MCPA	75.0%	77.4%
	2,4-DP	96.0%	77.4%

Fig. 2. Tanimoto scores for MCPP, 2,4-DP and MCPA using the MACCS keys software.

functional observation battery and motor activity monitoring. Food consumption was significantly lower throughout the study in males and in females during the last week of treatment. The same was found regarding body weights and body weight changes. There were no statistically significant changes in hematological parameters. Clinical chemistry revealed several changes with relatively

small magnitude. These included higher alanine aminotransferase activity and magnesium levels and lower bilirubin, glucose and albumin levels in females and higher creatinine and lower glucose levels in males. Urinalysis revealed a slightly increased urobilinogen excretion in males and females significant only for females. An increased number of degenerated transitional epithelial kidney

Table 1

Summary of acute toxicity of the two source substances MCPP and 2,4-DP and the target substance MCPA. Findings are based on the toxicological studies described in [USA- EPA \(2007a\)](#) and [California Environmental Protection Agency \(1999\)](#) for MCPP, [USA-EPA \(2007b\)](#) for 2,4-DP and [USA-EPA \(2004\)](#) for MCPA.

	MCPP	2,4-DP	MCPA
Acute oral toxicity (mg/kg bw)	LD50 = 775	LD50 = 567	LD50 = 765
Acute inhalation toxicity (mg/l air)	LC50 > 5.6	LC50 > 2.3	LC50 > 6.3
Acute dermal toxicity (mg/kg bw)	LD50 > 2000	LD50 > 2000	LD50 > 2000
Skin irritation	Irritant	Irritant	Slightly irritant (no classification)
Eye irritation	Strongly irritant	Strongly irritant	Strongly irritant
Skin sensitization	Non sensitizer	Non sensitizer	Non sensitizer
Mutagenicity	Overall negative	Overall negative	Overall negative

Table 2

Summary of ADME data of the two source substances MCPA and 2,4-DP and the target substance MCPP. Findings are based on the toxicological studies described in [USA- EPA \(2007a\)](#) and [California Environmental Protection Agency \(1999\)](#) for MCPP, [USA-EPA \(2007b\)](#) for 2,4-DP and [USA-EPA \(2004\)](#) for MCPA.

	MCPP	2,4-DP	MCPA
Absorption	>83%	>94%	>95%
metabolism	hydroxylation (parent and hydroxylated metabolites > 93%)	minor	undefined (parent 71%, undefined metabolite fraction 14%)
Elimination (urine)	>80%	>85%	>86%
Elimination (feces)	<13%	<7%	<7%
Half life	<8 h	<8 h	4–8 h

Table 3

Summary of 28d toxicity data of the two source substances MCPA and 2,4-DP and the target substance MCPP. Findings are based on the toxicological studies described in [van Ravenzwaay et al., 2005](#) (MCPA), [California Environmental Protection Agency, 2002](#) (2,4-DP) and [European Commission 2003](#) (MCPP).

	MCPP		2,4DP		MCPA	
Dose level	50 and 400 ppm		100 or 500 ppm		2000 ppm	
Sex	Male	Female	Male	Female	Male	Female
Findings Food consumption and body weight	–	–	–	–	Reduced food consumption and body weight development	Reduced food consumption and body weight development
Clinical Chemistry	Increased creatinine levels; Decreased glucose levels	Increased alanine aminotransferase activity and magnesium levels; Decreased bilirubin, glucose and albumin levels	–	–	Decreased cholesterol levels	Increased urea and creatinine levels; Decreased cholesterol levels
Urinalysis	Increased urobilinogen excretion (not significant); Increased number of degenerated transitional epithelial cells	Increased urobilinogen excretion	–	–	–	–
Necropsy	Lower absolute weights of liver, epididymides, heart, spleen, thymus and adrenal glands; Higher relative weights of testes and brain; (multi)focal tubular degeneration, occurring in single tubules of testes (3/5)	lower absolute weights of thymus and lower absolute and relative weights of ovaries and adrenal glands; slightly decreased number of hematopoietic cells was noted in the bone marrow (2/5)	–	–	Increased kidney weight liver weights (not considered adverse)	Increased kidney weight liver weights (not considered adverse)
NOAEL	400 ppm		Not determined		Not determined	
NOEL	50 ppm		500 ppm		Not determined	

cells in three of five males receiving MCPA was observed. At necropsy, males showed significantly lower absolute weights of liver, epididymides, heart, spleen, thymus and adrenal glands and significantly higher relative weights of testes and brain. Significantly lower absolute weights of thymus and lower absolute and relative weights of ovaries and adrenal glands were recorded for females. The incidence of gross organ findings did not distinguish treated animals from controls. A slightly decreased number of hematopoietic cells was noted in the bone marrow of two females. A (multi)focal tubular degeneration, occurring in single tubules, was observed in the testes of three males.

Overall, MCPA at 2000 ppm caused a reduction in body weight, which explains the lower absolute organ weights. Clinical chemistry suggests that liver and kidney are target organs at a functional

level, but doses were not high enough to cause pathological changes. The testes may be an additional target organ, although the observed changes may also be related to the decreased body weights in these young adult animals. A NOAEL was not determined in this study.

3.2. 2,4-DP (reviewed by [California Environmental Protection Agency, 2002](#))

Ten male and ten female Wistar rats per group received 2,4-DP at a dietary concentration of 0, 100 or 500 ppm of 2,4-DP for 4 weeks (OECD 407 guideline study). The test substance intake corresponded to 9 and 42 mg/kg/day in males and 9 and 45 mg/kg/day in females.

No mortality resulted from the treatment. There were no treatment-related clinical signs nor effects upon mean body weight, food consumption, hematology or clinical chemistry. Necropsy and histopathology did not reveal any treatment-related lesions. The NOEL (M/F) was determined to be 500 ppm.

3.3. Conclusion 28-day toxicity source substances

The NOEL for MCPA is below 170 mg/kg bw/day. At this dose body weight development was affected, and a functional impairment of the liver and the kidney was suggested by clinical chemical/urinalysis parameters and potentially testicular toxicity. The NOEL for 2,4-DP is above 42 mg/kg bw, a toxicity profile was not obtained. Although no definitive toxicity data for 2,4-DP were obtained in the 28-day study, there is no formal disagreement between the two source substances. Given the similarity in structure it could be proposed that the toxicity may be similar.

3.4. MCPP (reviewed by European commission 2012)

Ten male and female Wistar rats were fed a diet containing 0, 50 and 400 ppm MCPP (ca. 5 mg/kg bw and 36 mg/kg bw respectively). The 28-day study (according to OECD407 guidelines) was extended by 21 days, making the total study duration 49 days (7 weeks). Body weights and food consumption were not affected by treatment. At 400 ppm there was a reduction in cholesterol levels in male and female animals, as well as an increase in urea and creatinine values in female rats. At 400 ppm increased kidney weight and increased liver weights were seen in male and female animals. These changes were not considered to be adverse in nature. The NOAEL was considered to be 400 ppm, while the NOEL in this study was 50 ppm (ca. 5 mg/kg bw/day).

4. Metabolomics

4.1. Metabolome analysis

Within the context of metabolomics, metabolites are defined as small endogenous compounds such as carbohydrates, amino acids, nucleic acids or fatty acids and their derivatives resulting from biochemical pathways (Lindon et al., 2004, 2006). The use of sensitive LC-MS and GC-MS (gas chromatography coupled with mass spectrometry) techniques offers the possibility to detect a broad range of such metabolites and thus increases the chance of finding relevant biomarkers or patterns of change associated with a biochemical effect. We have shown that metabolite profiling in rats may well serve as a tool for identification of toxicological modes of action (Mattes et al., 2013, 2014; Kamp et al., 2012; Strauss et al., 2009; van Ravenzwaay et al., 2007). Since 2004, BASF SE has established a large metabolomics database (MetaMap[®]Tox) for data-rich chemicals, agrochemicals and drugs.

Wistar rats were maintained in an air-conditioned room under standardized environmental conditions. Dose levels for MCPP, MCPA and 2,4-DP were 1000 and 2500 ppm. For each dose group five male and five female animals were used. The common control group consisted of 10 males and 10 females. For all compounds tested blood samples were taken after 7, 14 and 28 days at the same period of time in order to avoid changes related to circadian rhythms. The study design can be best compared to an OECD 407 guideline design with two dose levels and can be easily integrated in this type of guideline study. However, in this specific setup only the metabolome is analyzed and no further examinations (e.g. pathology, clinical chemistry) was conducted.

The plasma metabolome was examined by GC-MS and LC-MS/MS techniques, as described in van Ravenzwaay et al., 2007.

Briefly, proteins were removed from plasma samples by precipitation. Subsequently, polar and non-polar fractions were separated for both GC-MS and LC-MS/MS analysis by adding water and a mixture of ethanol and dichloromethane. For GC-MS analysis, the non-polar fraction was treated with methanol under acidic conditions to yield the fatty acid methyl esters derived from both free fatty acids and hydrolyzed complex lipids. The non-polar and polar fractions were further derivatized with O-methyl-hydroxylamine hydrochloride and pyridine to convert oxo-groups to O-methyl-oximes and subsequently with a silylating agent before analysis (Roessner et al., 2000). For LC-MS analysis, both fractions were reconstituted in appropriate solvent mixtures. HPLC was performed by gradient elution using methanol/water/formic acid on reversed phase separation columns. Mass spectrometric detection technology was applied which allows target and high sensitivity MRM (Multiple Reaction Monitoring) profiling in parallel to a full scan analysis (European Patent application no.: 03711878.3; PCT/EP2003/001274). Following comprehensive analytical validation steps, the data for each analyte were normalized against data from pool samples. These samples were run in parallel through the whole process to account for process variability. For all metabolites, changes were calculated as the ratio of the mean of metabolite levels in individual rats in a treatment group relative to mean of metabolite levels in rats in a matched control group (time point, dose level, sex). The data generated were analyzed by univariate and multivariate statistical methods and a sex and day-stratified heteroscedastic *t*-test (“Welch test”) was applied to compare treated groups with respective controls. P-values and ratios of corresponding group medians were collected as metabolic profiles and fed into the MetaMap[®]Tox database.

4.2. Metabolome data evaluation

4.2.1. Pattern ranking

Based on the hypothesis that chemicals or diseases that produce a specific form of toxicity through a shared mode of action (MOA), should produce at least a subset of specific metabolite changes which are the same for all of these compounds, patterns of metabolite changes associated with toxicity were established. For the creation of such patterns, we have defined specific rules (van Ravenzwaay et al., 2014). We have set a threshold for selectivity in such a way, that compounds which match at least with 75% of the metabolite regulations in the established patterns fit to the MOA (>75% = weak match; > 90% = match, see Tables 4–8). The metabolite profile of a compound under investigation (here: MCPP, MCPA and 2,4-DP) is compared, by an automated procedure, against all of the patterns associated with a particular MOA or adverse outcome.

The pattern ranking for itself is a two-step process. Firstly, an algorithm used in the database compares the metabolite profile of a compound under investigation against all of the patterns associated with a particular mode of action or adverse outcome. This comparison yields a ranking list based on similarity of the test compound metabolite profile compared to the specific patterns in MetaMap[®]Tox, using a median *r*-value metric. Secondly, the metabolite changes are evaluated by an expert panel of experienced toxicologists to determine what may be described as “confirmed” matches. Generally, based on the number of commonly changed metabolites, a match requires that approx. 90% or more of metabolites significantly changed as defined by the pattern (weak matches: approx. 75–90%). Furthermore, the quality and importance of the metabolite changes for a certain toxicological mode of action are considered for this evaluation. For example, metabolites which are based on perturbances of specific biochemical pathways, and which can be connected to the toxicity

Table 4

Pattern ranking of MCPA demonstrated the following matches/weak matches with pre-defined patterns in the database.

Dose group	Sex	Pattern - Mode of Action	Assessment
2500 ppm	Male	Kidney inhibition weak organic acids	Match
		Liver peroxisome proliferation	Match
		Liver fibrate phthalate phenoxy	Match
		Phthalates long chain	Match
		Reduced feed consumption	Match
		Liver PPAR gamma agonist	Weak match
	Female	Kidney inhibition weak acid	Match
		Liver peroxisome proliferation	Weak match
1000 ppm	Male	Liver oxidative stress	Match
		Liver peroxisome proliferation	Weak match
		Liver fibrate, phthalate, phenoxy	Weak match
		Reduced feed consumption	Weak match
	Female	Kidney inhibition weak acid transport	Match

Match
Weak match

Table 5

Pattern ranking of 2,4-DP demonstrated the following matches/weak matches with pre-defined patterns in the database.

Dose group	Sex	Pattern - Mode of Action	Assessment
2500 ppm	Male	Liver peroxisome proliferation	Match
		Liver fibrate phthalate phenoxy	Match
		Kidney inhibition weak acid	Match
		Phthalates long chain	Weak match
		Liver oxidative stress	Weak match
	Female	Liver peroxisome proliferation	Match
1000 ppm	Male	Kidney inhibition weak acid	Match
		Liver fibrate phthalate phenoxy	Weak match
		Reduced feed consumption	Weak match

Match
Weak match

Table 6

Pattern ranking of MCPP demonstrated the following matches/weak matches with pre-defined patterns in the database.

Dose group	Sex	Pattern - Mode of Action	Assessment
2500 ppm	Male	Liver peroxisome proliferation	Match
		Liver fibrate phthalate phenoxy	Match
		Phthalates long chain	Match
		Reduced feed consumption	Match
		Kidney inhibition weak organic acids	Match
		Liver oxidative stress	Weak match
		Liver PPAR alpha agonist	Weak match
	Female	Liver peroxisome proliferation	Match
1000 ppm	Male	Kidney inhibition weak acids	Match
		Liver peroxisome proliferation	Match
		Liver fibrate phthalate phenoxy	Match
		Liver PPAR gamma agonist	Weak match
		Phthalates long chain	Weak match
	Female	Liver peroxisome proliferation	Weak match

Match
Weak match

observed, are particularly evaluated. For some patterns, we have defined so-called “anchor metabolites”. These metabolites are essential components of the biochemical pathway related to the mode of action, and these have to be significantly changed in the right direction in order to result in a matching pattern. For those patterns for which a match (or close match) is noted, a subsequent

expert judgement of the individual metabolite regulations is performed.

4.2.2. Profile comparison

The profile comparison is performed on the basis of the entire metabolome profile of a target compound, against all other

Table 7

Common patterns obtained in the metabolome analysis with the source substances MCPA and 2,4-DP and MCPP in male animals treated with 2500 ppm.

Mode of action	2,4-DP	MCPA	MCPP
Liver peroxisome proliferation			
Liver fibrates phthalate and phenoxy			
Reduced feed consumption	-		
Kidney inhibition weak org. acids			
Phthalates long chain			
Liver PPAR alpha agonist			
Liver oxidative stress		-	

	Match
	Weak match

Table 8

Common patterns obtained in the metabolome analysis with the source substances MCPA and 2,4-DP and MCPP in female animals treated with 2500 ppm.

Pattern	2,4-DP	MCPA	MCPP
Liver peroxisome proliferation			
Kidney inhibition weak acids			

	Match
	Weak match

metabolite profiles available in the database using Spearman and Pearson correlations. The result is a ranking list of compounds most similar to the new compound arranged by the correlation factor. The Pearson correlation as the default option takes into account both the overall quality of the matches of metabolite regulation, as well as the strength of profoundly regulated metabolites. As nearly all of the compounds in the database have a well-known toxicological profile, the best matches are informative of the type of toxicity that may be expected. In order to assess the size and relevance of a correlation coefficient a reference distribution of correlation coefficients was derived by calculating all pairwise coefficients of the whole database stratified by sex (male/female) and dose (high/low). As each stratum comprises approximately 500 treatments (t-profiles) the quantiles of each reference distribution are based on approximately 130,000 r-values/stratum. Based on these analyses, a threshold value of 0.40 for male animals and 0.50 for female animals displays approximately the 95th percentile of all correlation coefficients obtained by the profile comparison. Correlation coefficients above these values are considered as indicating a clear match between two treatments and as likely to be biologically relevant.

4.2.3. Biochemical pathways

With metabolomics the regulation of several metabolites in blood is assessed. For the establishment of the MetaMap[®]Tox

database the metabolite changes are interpreted together with clinical pathology and histopathology data. This information can be used to explain biochemical pathway alterations of unknown compounds.

4.2.4. Expert judgement

Final conclusions are drawn based on a multi-step procedure. Firstly, an inspection of all regulated metabolites is performed and possible conclusions with respect to biochemical pathways are drawn. Following steps are pattern ranking and profile comparison. It is essential to combine the results of all analyses before conclusions are drawn. When the identified profiles match with the toxicological properties of compounds which have a high rank in the pair-wise comparison, there is reasonable certainty that a correct MOA/adverse outcome has been identified. The results of a compound analysis are always presented to a team of experts.

4.3. Strengths and weaknesses of plasma based metabolomics

Metabolome changes are the last in line of a series of changes caused by toxicity or the adaption of an organism to new conditions. Not all DNA is transcribed into RNA, not all RNA is turned into protein, and not all protein expressed is active. However, if this cascade of changes does occur and e.g. more P450 enzymes are formed, then this increased activity will affect the metabolites of a

cell. Thus, metabolite changes are the last step before classical toxicological observations manifest themselves.

Plasma was selected as the matrix for analysis because all organs exchange metabolites with the blood, and as such one matrix could serve as a melting pot for all changes in an organism. The advantage is that this significantly reduces the amount of testing necessary, the sample procedure is only minimally invasive and that animals can be followed longitudinally over time. The disadvantage is that for compounds with multiple organ toxicities the changes in metabolites cannot be immediately allocated to an organ, and biochemical pathways are in many cases not easy to determine. It should also be noted that the plasma based metabolomics as presented here is not a high throughput technology. For building our database we have selected time points (day 7, 14 and 28 of administration) for which we assume that already a certain level of homeostasis is achieved within the organism. The database is limited to systemic toxicity testing and applicable for short- and subchronic-toxicity evaluations. The obtained MOA information is helpful to address other points such as human relevance (if known for a particular MOA) or to identify potential effects on fertility. It is not suitable, however, for developmental toxicity, and more subtle forms of reproduction toxicity.

The database now contains more than 500 reference compounds and a further 250 compounds with limited toxicological data. The very high number of reference compounds has allowed us to create about 120 patterns of metabolomic changes that can be associated with nearly all of the classical forms of organ toxicity observed in OECD guideline studies for systemic toxicity. Over the last years we have compared our prediction based on metabolomics with that of classical pathology in the same study. Overall, the rate of correct prediction was >80% (manuscript in preparation). In a further analysis of 122 cases, we have looked at the relative sensitivity of metabolomics versus classical toxicology (van Ravenzwaay et al., 2014). Again in >80% of these cases, sensitivity was similar (i.e. we observed metabolome changes at effect levels in the classical study and the absence of effect levels where a NOAEL had been determined). Increased sensitivity of metabolomics (12% of all cases) was noted for enzyme changes (e.g. liver enzyme induction). In the absence of further findings, such changes are not regarded as adverse, thus largely explaining the apparent increased sensitivity. The cases of reduced sensitivity (accounting for 5% of all cases) can be allocated to three groups:

1. Organs for which no pattern has been established yet (i.e. rare forms of toxicity) but for which a metabolome change is visible, one case of sertoli-cell toxicity.
2. Organs for which no pattern has been established yet (i.e. rare forms of toxicity) and for which a metabolome change is not visible, one case of crystals in the urinary bladder
3. Reduced body weight during the first week of administration (related to initially reduced food consumption) with a subsequent recovery. For these studies only day 28 blood was analyzed.

As mentioned earlier metabolite changes observed in the blood may be associated with more than one form of organ toxicity. In establishing selective patterns, we dissect the overall changes into separate patterns. This is achieved by 1) using compounds with very specific effects (single organ toxicity) and establishing patterns for selective organ toxicity. In case of mixture effects, these patterns can be subtracted from the overall changes, and the remaining changes are then further analyzed. A further way to obtain a selective profile is to combine the evaluation for many compounds, which share a particular form of toxicity, and to request that only those metabolites are shown which are

commonly regulated in all compounds. If the number of compounds is high enough, only the common MOA will remain.

It should be noted that the strength of changes in a particular profile cannot be immediately correlated with the strength of the toxicity, because certain organs (e.g. liver) have a more dominating effect on the plasma metabolome than others (e.g. lung).

5. Results of the metabolome analysis

5.1. Pattern ranking

5.1.1. Evaluation of MCPA

MCPA was administered for 28 days to Wistar rats at dose levels of 1000 and 2500 ppm. Blood samples were taken at day 7, 14 and 28 for metabolome analysis.

At the high dose level, body weight was significantly decreased in male (17%) and female (15%) animals most likely due to a significantly reduced food consumption of up to 45% compared to controls in female animals and up to 21% in male animals. Neither body weight nor food consumption was affected by the low-dose treatment. The matches/weak matches with pre-defined patterns obtained for metabolome pattern ranking of MCPA are depicted in Table 4.

Based on these matches, MCPA, dosed at 2500 ppm is expected to have the liver and the kidney as its target organs. Expected MOA of toxicity are induction of biochemical parameters related to fatty acid metabolism, potentially resulting in peroxisome proliferation as well as reduction in the capacity of the kidney to transport weak organic acids. The likelihood of the latter corollary is increased taking into account the structure of MCPA (this compound can be considered as a weak organic acid).

At 1000 ppm, in males the liver is expected to be the target organ, in females the kidney would be predicted to be the more sensitive organ. A NOAEL below 1000 ppm would be expected. Comparing these results with the available data from the 28-day study performed with this compound shows that the predicted MOA/target organs are in line with the toxicological results.

5.1.2. Evaluation of 2,4-DP

2,4-DP was administered for 28 days to Wistar rats at dose levels of 1000 and 2500 ppm. Blood samples were taken at day 7, 14 and 28 for metabolome analysis.

The body weight in male animals treated with 2500 ppm of 2,4-DP was significantly decreased up to 13% after 28 days although food consumption was only decreased at the beginning of the treatment (26%). In female animals, body weight was significantly decreased up to 10% after 28 days of treatment although food consumption was not affected by the treatment. Male animals treated with the low dose of 2,4-DP showed a slight increase in body weight due to increased food consumption, whereas body weight and food consumption of female animals were unaffected. The latter changes are not considered to be adverse in nature, however. The matches/weak matches with pre-defined patterns obtained for metabolome pattern ranking of 2,4-DP are depicted in Table 5. Based on these matches 2,4-DP when dosed at 2500 ppm is expected to have the liver and the kidney as its target organs. Expected MOAs of toxicity are induction of biochemical parameters related to fatty acid metabolism, resulting in peroxisome proliferation as well as reduction in the capacity of the kidney to transport weak organic acids. 2,4-DP based on its structure can be considered as a weak organic acid. At 1000 ppm the liver in males can be expected as a target organ.

As the 28-day toxicity study with 2,4-DP was dosed at significantly lower dose levels than those used in the metabolome study, there is no possibility to verify the results of the metabolome

prediction with those of the classical 28-day toxicity study in rats. It should be noted here, however, that the predicted MOA of MCPA and 2,4-DP are substantially similar and that body weight development at 2500 ppm for both compounds in the metabolome studies was reduced.

5.1.3. Evaluation of MCPP

MCPP was administered for 28 days to Wistar rats at dose levels of 1000 and 2500 ppm. Blood samples were taken at day 7, 14 and 28 for metabolome analysis.

The body weight of male animals treated with 2500 ppm MCPP was decreased up to 12% after 28 days of treatment although food consumption was only significantly decreased (11%) at the beginning of the treatment. Female animals showed a reduction of body weight of up to 14% at the end of the treatment in accordance with a significant reduction of food consumption of up to 31%. Male animals treated with the lower dose of MCPP showed a significant increase in food consumption at the end of the treatment, however the body weight was not affected. Body weight and food consumption of female animals treated with the lower dose of MCPP were not affected by the treatment. The matches/weak matches with pre-defined patterns obtained for metabolome pattern ranking of MCPP are depicted in Table 6. Based on these matches MCPP when dosed at 2500 ppm is expected to have the liver and the kidney as its target organs. Expected MOAs of toxicity are induction of biochemical parameters related to fatty acid metabolism, possibly resulting in peroxisome proliferation as well as reduction in the capacity of the kidney to transport weak organic acids. Based on the chemical structure of MCPP, this compound can be considered as a weak organic acid.

As the 28-day toxicity studies with MCPP were dosed at significantly lower dose levels there is no possibility to exactly verify the results of the metabolome prediction with those of the classical 28-day toxicity study in rats. The findings observed in the 28-day study at 400 ppm (1) reduction in cholesterol and increased liver weight suggest the liver to be a target organ, and (2) an increase in urea and creatinine values and increased kidney weight suggest the kidney to be the second target organ. This is exactly in line with the predictions from the metabolome investigation, albeit at higher dose levels. It should be noted here, that the predicted MOAs of MCPP are substantially similar with those of MCPA and 2,4-DP, and that at 2500 ppm all compounds affect body weight development in a similar way.

5.1.4. Combined metabolome evaluation

All three compounds showed a clear effect on the liver, matching patterns for liver peroxisome proliferation and fibrates as well as phthalate induced liver toxicity, the MOA of these compounds being related to a lipid reducing effect, based on PPAR- α induction and subsequent peroxisome proliferation (Table 7). 2,4-DP and MCPP showed also weak matches for liver oxidative stress, whereas MCPA and MCPP showed a weak match indicating activation of the liver PPAR receptor. In addition to the liver, also the kidney was identified as a target organ. All treatments generated at least a weak match with the pattern for the inhibition of the transport of weak organic acids in the kidneys. MCPA and MCPP also matched with a pattern for reduced feed consumption. Analysis of the clinical data confirmed this finding for all three substances. This also led to a reduction of body weight of up to 17% during treatment. Overall, the three treatments showed a good overlap regarding the toxicity MOA patterns generated.

In female animals, the target organs were also liver and kidney, but less pronounced compared to male animals. All three treatments resulted in matches for liver peroxisome proliferation and the inhibition of weak acid transportation in kidneys (Table 8).

5.2. Profile comparison

If all treatments present in MetaMap[®]Tox were correlated with MCPP (more than 1800 treatments, more than 800 different substances) it correlated best with itself in a repeated study in male animals, which was conducted to evaluate the reproducibility of the test system. 2,4-DP is directly ranked at the second position with a very high correlation coefficient of 0.79. In female animals MCPP correlated best with 2,4-DP with a very high correlation coefficient of 0.82. These values correspond to the 99th percentile in males and in females of all possible rankings, clearly showing the very high similarity between the two compounds. Although the similarity percentile of MCPA with MCPP still is quite high (>95th percentile), it did not rank as close as 2,4-DP did. Compounds with a better overall metabolome match than MCPA were fibrates and phthalates. The common denominator of these substances is the induction of lipid metabolism and the potential to cause peroxisome proliferation. As this is also one of the two dominant MOAs identified for MCPP (as well as 2,4-DP) this good match is not surprising, but rather a confirmation of the conclusions drawn from the pattern ranking. Overall, it can be concluded that the profile comparison of the compounds is in good accordance with the predicted toxicity profiles of the compounds. Profile comparison confirms the indication obtained from evaluation of the metabolites, that 2,4-DP is more similar to MCPP than MCPA, and thus would be a more suitable source substance. The latter can also be visualized by a principal component analysis depicted in Fig. 3.

5.2.1. Quantitative prediction of toxicity

The results of the 90-day toxicity study with 2,4-DP are fully in line with what could be expected based on the 28-day metabolome analysis, and the data obtained in the 28-day study. At 2500 ppm there were effects on body weight, and the target organs were the liver and the kidney. As mentioned above, from a metabolomics (biological) point of view, MCPP is more closely related to 2,4-DP than to MCPA. Thus we would predict the same qualitative toxicity profile for MCPP as that for 2,4-DP.

The metabolome analysis can also be used to provide a comparative estimate of the strength of the toxicity induced treatments. There are two ways to obtain such quantitative information. On the one hand, the strength of effect can be estimated by the sum of the fold change values of commonly, significantly changed metabolites ($p < 0.05$, see Table 1) and compared with that of the same dose (2500 ppm) of the three compounds under investigation (Table 9). This calculation was also performed for the commonly, significantly changed metabolites when a lower dose (1000 ppm) was administered.

Overall, the compounds appear to be equally potent. Females have lower values than males. There is a steep dose relationship, and the values found at 1000 ppm are only moderately higher than what could be expected from random change. A further way to quantitatively compare the profiles of the three compounds is to calculate the overall profile strength. The overall profile strength of a “target profile” is calculated as the median profile P of all analytes of target treatment:

$$P = (p_1, p_2, \dots, p_n), p_x \neq na \quad S_p = \frac{\sum_{k=1}^n |p_k|}{n}$$

In short, it can be described as the “rounded down average of absolute medians of t-values”. The advantage of this second method is that it takes all metabolites into account and is independent on an arbitrary set p-value. Results of these calculations are shown in Table 10. The results of the evaluation of the overall profile strength indicate that MCPP and MCPA have a similar

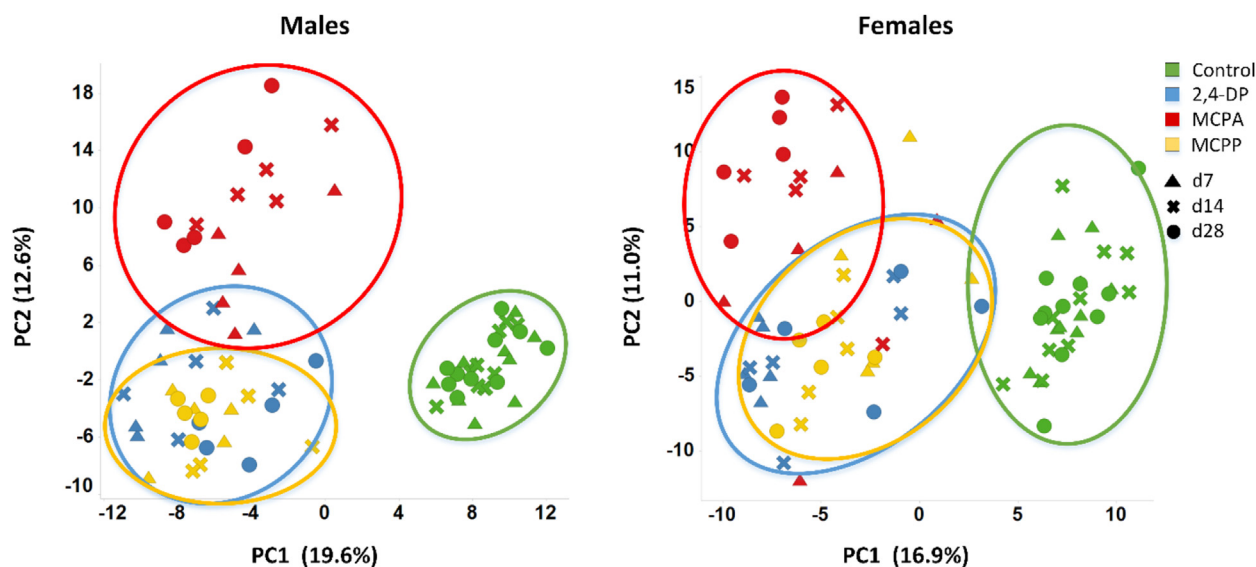


Fig. 3. Principle component analysis of the metabolome of MCPA, 2,4-DP and MCPP. Overall profiles are similar, however, subtle compound differences can be differentiated. The metabolic profile produced by MCPP is more similar to the one produced by 2,4-DP than to the metabolic changes induced by MCPA.

Table 9

Sum of the fold changes of commonly significantly changed metabolites ($p < 0.05$) at three time points in rat plasma of four week studies with administration of 2500 ppm 2,4-DP, MCPP and MCPA and with administration of 1000 ppm of the three compounds. For all fold changes < 1.0 reciprocal values were used for calculation of the sums.

Dose	2,4-DP		MCPP		MCPA	
	Males	Females	Males	Females	Males	Females
2500 ppm	292	210	292	165	320	185
1000 ppm	29	23	33	23	30	33

strength of effect, whereas 2,4-DP is slightly weaker. The dose response relationship is relatively steep, the values obtained for 2,4-DP are suggestive of only a moderate effect and approaching those of control values (control values/random change values go up to a value of ca. 0.8). The values at 1000 ppm for MCPP and MCPA are indicative of a clear test substance related effect at this dose level.

The overall quantitative analysis of the strength of effects indicates that there are no great differences between the three compounds. MCPP is at least as potent as 2,4-DP and possibly slightly stronger, particularly at the lower dose level. For read across purposes, we assume the same strength of effects at the high dose level. We predict that the NOAEL for MCPP may be below the one for 2,4-DP. Thus, in conclusion, we expect the 90-day toxicity of MCPP to be similar to that of 2,4-DP, however, with potentially a lower NOEL (comparable to that of MCPA).

Table 10

Overall profile strength of 2,4-DP, MCPP and MCPA for males and females at 2500 ppm and 1000 ppm.

Dose	2,4-DP		MCPP		MCPA	
	Males	Females	Males	Females	Males	Females
2500 ppm	2.44	2.29	3.02	2.84	3.01	2.79
1000 ppm	1.14	1.1	1.85	1.57	1.81	1.71

5.3. Biochemical pathways

The magnitude of metabolic changes induced by all three substances (determined at $p < 0.05$) were comparable. In male animals of the high dose groups (2500 ppm) between 35 and 43% of the measured metabolites were significantly changed, in females of the mentioned dose groups 24–40%, which represents a strong effect of the three substances on the metabolome. Especially in males, a large subset of metabolites was regulated in common due to treatment with the test substances (Table 11).

These included, amongst others, amino acids and a large fraction of fatty acids, phosphatidylcholines and lysophosphatidylcholines. In females, not as many metabolites were regulated in common by all three treatments. Uniquely, in both sexes down-regulated metabolites are long chain fatty acids ($> C18$), i.e. 17-methyloctadecanoic acid and docosahexaenoic acid, whereas specifically dihomogamma-linolenic acid is up-regulated in males and females (Table 11 and Table 12). These changes are induced typically by peroxisome proliferators. It can be assumed that the common glucuronic acid increase in plasma of male and female rats is an effect of the inhibition of the renal excretion due to the compounds. A unique downregulation of special amino acids (e.g., tryptophan, methionine, lysine and proline) are a hint of a common influence of the compounds on the metabolism of these amino acids. In contrast, various triacylglycerides and phosphatidylcholines were up-regulated in females but down-regulated in male animals. This contrasting sex-specific effect on lipids can be often observed.

In Table 13 metabolites in male animals are listed that are commonly regulated due to treatment with two of the test substances, but not the third. MCPP and 2,4-DP share more commonly regulated metabolites, than MCPA. The same is depicted for female animals in Table 14. The fact that there are more metabolites commonly regulated between MCPP and 2,4-DP than between MCPP and MCPA is further evidence indicating that 2,4-DP is more suitable as a source substance than MCPA for read across to the target substance MCPP.

Table 11

Commonly regulated metabolites for all three substances in male animals treated with 2500 ppm of the test compound.

Metabolite	2,4-DP			MCPA			MCPP		
	m7	m14	m28	m7	m14	m28	m7	m14	m28
16-Methylheptadecanoic acid	0,24	0,31	0,41	0,23	0,33	0,18	0,23	0,25	0,21
17-Methyloctadecanoic acid	0,22	0,34	0,30	0,29	0,35	0,20	0,16	0,24	0,16
3-Hydroxyindole	3,70	3,54	3,94	1,95	2,58	2,93	2,59	2,56	1,94
Arachidonic acid (C20:cis[5,8,11,14]4)	0,20	0,29	0,41	0,27	0,42	0,26	0,28	0,34	0,26
Arginine	0,74	0,80	0,68	0,79	0,73	0,76	0,78	0,82	0,67
Asparagine	0,62	0,74	0,66	0,75	0,59	0,74	0,74	0,72	0,72
Cholesterylester C20:4	0,21	0,21	0,35	0,57	0,29	0,33	0,29	0,33	0,44
Cytosine	0,44	0,62	0,69	0,63	0,60	0,60	0,73	0,73	0,66
dihomo-gamma-Linolenic acid (C20:cis[8,11,14]3)	3,67	3,48	2,79	3,87	6,34	8,21	2,58	2,99	3,44
Docosahexaenoic acid (C22:cis[4,7,10,13,16,19]6)	0,15	0,21	0,23	0,15	0,20	0,09	0,17	0,24	0,15
Docosapentaenoic acid (C22:cis[7,10,13,16,19]5)	0,23	0,21	0,16	0,15	0,25	0,13	0,20	0,30	0,21
Glucuronic acid	6,79	5,82	3,32	3,06	2,88	3,87	4,49	3,48	2,27
Ketoleucine	0,57	0,62	0,62	0,39	0,26	0,34	0,72	0,79	0,57
Lysine	0,44	0,52	0,56	0,40	0,30	0,33	0,57	0,60	0,50
Lyso PE (C22:0) (putative)	0,24	0,21	0,28	0,38	0,28	0,29	0,20	0,20	0,18
Lysophosphatidylcholine (C17:0)	0,43	0,35	0,35	0,59	0,54	0,35	0,43	0,34	0,24
Lysophosphatidylcholine (C18:0)	0,77	0,78	0,83	0,81	0,83	0,73	0,75	0,78	0,77
Lysophosphatidylcholine (C18:2)	1,28	1,47	1,05	1,54	1,40	1,39	1,38	1,40	1,24
Methionine	0,76	0,73	0,81	0,66	0,59	0,64	0,72	0,82	0,80
PC No 04 (putative)	0,28	0,37	0,30	0,42	0,44	0,36	0,30	0,40	0,34
Phosphatidylcholine (C16:0,C20:4)	0,71	0,74	0,77	0,63	0,80	0,62	0,62	0,67	0,64
Phosphatidylcholine (C16:0,C20:5)	1,48	1,51	1,19	1,73	1,82	2,11	1,43	1,20	1,22
Phosphatidylcholine (C16:0,C22:6)	0,46	0,44	0,50	0,37	0,45	0,34	0,40	0,39	0,38
Phosphatidylcholine (C18:0,C20:3)	0,53	0,46	0,53	0,49	0,82	0,48	0,37	0,47	0,38
Phosphatidylcholine (C18:0,C20:4)	0,36	0,40	0,51	0,36	0,55	0,24	0,32	0,41	0,38
Phosphatidylcholine (C18:0,C22:6)	0,34	0,38	0,41	0,30	0,30	0,18	0,29	0,33	0,30
Phosphatidylcholine No 02	0,43	0,37	0,39	0,53	0,56	0,51	0,41	0,41	0,35
Proline	0,69	0,72	0,77	0,63	0,51	0,52	0,66	0,72	0,64
Pseudouridine	1,14	1,58	1,39	1,31	1,49	1,41	1,17	1,43	1,32
Stearic acid (C18:0)	0,34	0,50	0,45	0,48	0,67	0,43	0,36	0,39	0,38
TAG (putative)	0,64	0,54	0,46	0,35	0,59	0,36	0,32	0,35	0,40
Threonine	0,56	0,68	0,82	0,68	0,63	0,69	0,65	0,68	0,77
Tryptophan	0,21	0,24	0,45	0,20	0,19	0,18	0,33	0,50	0,49
Unknown lipid (68000033)	0,58	0,56	0,67	0,45	0,49	0,42	0,57	0,54	0,56
Unknown lipid (68000034)	0,37	0,30	0,38	0,31	0,26	0,22	0,39	0,38	0,33
Unknown lipid (68000052)	0,31	0,33	0,48	0,31	0,42	0,22	0,29	0,31	0,29

6. Ninety-Day toxicity studies in rats

6.1. MCPA (reviewed by JMPR, 2012, Health Canada)

In a combined subchronic toxicity and neurotoxicity study (OECD 408 and 424 guideline, US-EPA OPTTS 870-3100 and 870-6200 guideline), MCPA was administered to groups of 15 male and 15 female Wistar rats for 3 months at dietary concentrations of 0, 50, 500 or 2500 ppm (equivalent to doses of 0, 3, 34 or 177 mg/kg bw per day for males and 0, 4, 42 or 188 mg/kg bw per day for females).

At the highest dose, a significant decrease in body weight development was observed in both sexes (42% in males, 48% in females), food consumption being clearly reduced particularly during the first 7 days of the study. At the highest dose, in both sexes, a significant decrease in hematological parameters (red blood cells, hemoglobin and hematocrit), and a significant increase

in liver enzymes (alanine aminotransferase, alkaline phosphatase and aspartate aminotransferase) were observed. Histopathology demonstrated alterations of hepatocytes, characterized by cytoplasmic eosinophilia and granular cytoplasm. In addition, a higher incidence/grading of foam cell accumulations in the lung and myeloid atrophy of the hematopoietic marrow were seen in both sexes. In high-dose males, a decrease in testes weights, testicular atrophy and atrophy of the seminal vesicles and prostate, aspermia or oligospermia in the epididymides were observed. For the neurotoxicity evaluation, functional observational battery and motor activity assessments were performed on 10 animals per sex per group prior to the treatment and on treatment days 22, 50 and 85. Effects observed at 2500 ppm were a decreased value of hindlimb grip strength in females on day 85, decreased foot splay test values ($p < 0.02$) in males on day 22 and reduced values ($p < 0.02$) of forelimb grip strength in males on day 50. The results of the FOB as well as those on testicular development need to be assessed

Table 12

Commonly regulated metabolites for all three substances in female animals treated with 2500 ppm of the test compound.

Metabolite	2,4-DP			MCPA			MCPP		
	f7	f14	f28	f7	f14	f28	f7	f14	f28
17-Methyloctadecanoic acid	0,51	0,60	0,60	0,43	0,32	0,26	0,39	0,37	0,45
dihomo-gamma-Linolenic acid (C20:cis[8,11,14]3)	7,68	7,97	4,96	3,78	5,28	6,14	3,91	6,17	8,56
Docosahexaenoic acid (C22:cis[4,7,10,13,16,19]6)	0,80	0,55	0,54	0,39	0,20	0,13	0,41	0,38	0,34
gamma-Linolenic acid (C18:cis[6,9,12]3)	6,14	6,64	3,31	4,23	2,93	2,99	3,03	3,62	4,04
Glucuronic acid	7,66	7,18	3,89	2,15	2,62	3,20	4,05	5,04	4,38
Glycerol, lipid fraction	3,12	2,68	2,19	1,69	1,38	1,60	1,96	2,17	2,15
Indole-3-lactic acid	0,16	0,22	0,32	0,24	0,24	0,23	0,60	0,30	0,43
Ketoleucine	0,44	0,45	0,34	0,43	0,18	0,16	0,52	0,57	0,54
Lysine	0,43	0,41	0,53	0,27	0,19	0,23	0,38	0,34	0,36
Methionine	0,90	0,83	0,79	0,81	0,60	0,61	0,83	0,82	0,82
Oleic acid (C18:cis[9]1)	2,81	3,10	2,62	1,75	1,73	1,86	2,00	2,47	2,54
Palmitic acid (C16:0)	2,53	2,69	1,57	1,56	1,26	1,31	1,56	1,90	1,99
Phosphatidylcholine (C16:0,C18:2)	1,47	1,51	1,29	1,66	1,82	1,45	1,25	1,58	1,34
Phosphatidylcholine (C18:0,C18:1)	1,36	1,43	1,44	1,57	1,74	1,56	1,23	1,33	1,40
Proline	0,85	0,79	0,75	0,61	0,45	0,41	0,64	0,72	0,66
Serine	0,82	0,83	0,73	0,69	0,59	0,61	0,80	0,82	0,81
TAG (C16:0,C18:1,C18:3)	19,55	11,95	3,97	7,58	4,41	3,79	6,43	8,72	6,34
TAG (C16:0,C18:2)	8,01	6,80	4,13	3,32	2,40	3,06	3,88	4,81	3,17
Tryptophan	0,17	0,20	0,31	0,17	0,18	0,16	0,50	0,36	0,44
Unknown polar (58000167)	1,78	1,64	1,81	1,63	1,81	1,85	1,95	2,15	2,37

keeping in mind that at the high dose level the maximum tolerated dose was clearly exceeded, both in terms of bodyweight development as well as in terms of being beyond the maximum capacity of renal excretion. Therefore, they may not be selective in nature. No significant treatment-related changes were seen at the two lowest doses. The NOAEL was at 500 ppm.

In a second study MCPA was administered to Wistar rats (15 per sex per dose) at dietary concentrations of 0, 50, 150 or 450 ppm (equivalent to doses of 0, 3.6, 10.9 and 32.6 mg/kg bw per day for males and 0, 4.0, 12.1 and 35.8 mg/kg bw per day for females).

At 450 ppm, an increase in creatinine values in the plasma of females was observed. At the same level, decreases in cholesterol and calcium values in the males were observed. Also, an increase in absolute and relative kidney weights in males was observed. At 150 ppm, increased absolute kidney weights (108% of controls) were noted ($p < 0.05$). No changes were observed at the lowest level (50 ppm). In the absence of histopathological changes, and clinical-pathological changes at 150 ppm, this dose could be regarded as a NOAEL.

The first study demonstrates that at 2500 ppm body weight development is severely affected. Target organs are the liver, as evidenced by clinical chemistry and histopathology. A second target is the hematopoietic system, demonstrated by reduced red blood cell values, probably caused by an atrophy of the hematopoietic bone marrow cells. Effects noted in the functional observation battery and the effects on the male reproductive system may have been secondary to the body weight effects. The second study, performed at lower dose levels primarily identifies the kidney as a target organ, as evidenced by increased kidney weights, urinary bilirubin, urinary crystals and altered pH values. The first study indicates a NOAEL at 500 ppm, whereas the second study suggests the NOAEL to be at 150 ppm.

6.2. 2,4-DP (reviewed by [California Environmental Protection Agency, 2002](#))

15 male and 15 female Wistar rats per group were dosed in the diet with 0, 100, 500, 2000 (males only) or 3000 (females only) ppm of 2,4-DP for 13 weeks in an OECD 408 like guideline study, corresponding to a test substance intake of 0, 7, 35, 144 mg/kg/day for males and 0, 8, 42, 245 mg/kg/day for females.

No mortality resulted from the treatment. No treatment-related signs were noted in the general clinical observations. The mean body weight of the 2000 ppm males was less than that of the controls during the first 8 weeks of the study ($p < 0.05$ or 0.01). Mean food consumption for these animals was less than that of the controls for the first 2 weeks of the study ($p < 0.05$ or 0.01). The mean body weight and food consumption values for the 3000 ppm females were lower than those of the control throughout the study ($p < 0.01$). In contrast, mean water consumption for the 2000 ppm males and the 3000 ppm females was greater than that of the controls throughout the study ($p < 0.01$). Only the fore and hind limb grip strength parameters were apparently affected in the functional observational battery. The mean values for these treated animals were all within the historical control range. Mean motor activity was reduced for the 3000 ppm females at the 5-day time point. Otherwise, no other apparent treatment-related effect on activity was noted. For hematology, red blood cells, hemoglobin, and hematocrit, values were less than those of the controls for both high dose males and females. Among the clinical chemistry parameters, mean serum alkaline phosphatase activity was increased for the 2000 ppm males and the 3000 ppm females. In addition, mean globulin, triglycerides, and cholesterol values were less than those of the controls for the high dose males and females. In the urinalysis results, specific gravity was lower and the presence of erythrocytes and bacteria was greater for the high dose females. No gross lesions were noted in the necropsy examination. Absolute

Table 13

Differentially regulated metabolites in phenoxy test substances in male animals. Listed are all metabolites which were commonly regulated in two of the test substances (MCP and 2,4-DP (a); MCP and MCPA (b)), but differentially regulated in the third substance.

a)	Metabolite	MCP			2,4-DP			MCPA		
		m7	m14	m28	m7	m14	m28	m7	m14	m28
	3-Indoxylsulfate	4,14	2,10	3,00	5,58	3,28	3,25	0,72	1,53	1,92
	3-Methoxytyrosine	1,33	1,35	1,76	1,22	1,32	1,35	1,08	1,19	1,84
	alpha-Tocopherol	0,56	0,65	0,59	0,70	0,63	0,68	0,93	1,09	0,98
	beta-Sitosterol	0,24	0,34	0,23	0,37	0,30	0,31	0,65	1,03	0,74
	Campesterol	0,30	0,36	0,23	0,31	0,29	0,32	0,68	1,04	0,99
	Cholesterol, total	0,44	0,50	0,45	0,38	0,48	0,52	0,67	0,90	0,73
	Ethanolamine plasmalogen (C39:4)	0,49	0,54	0,52	0,62	0,52	0,48	0,72	0,84	0,72
	Galactose, lipid fraction	0,52	0,51	0,56	0,62	0,45	0,65	0,65	0,90	0,86
	Indole-3-acetic acid	0,49	0,65	0,64	0,31	0,41	0,63	0,52	0,90	1,18
	myo-Inositol, lipid fraction	0,56	0,55	0,56	0,45	0,53	0,61	0,54	0,92	0,76
	myo-Inositol-2-phosphate, lipid fraction	0,18	0,22	0,25	0,27	0,21	0,32	0,30	0,61	0,52
	Myristic acid (C14:0)	0,61	0,81	0,58	0,61	0,72	0,44	0,53	0,71	0,81
	Pantothenic acid	3,57	4,54	4,58	2,45	3,34	3,73	0,92	1,41	0,86
	Phosphate, lipid fraction	0,64	0,74	0,67	0,64	0,69	0,62	0,75	1,01	0,80
	Sphingomyelin (d18:1,C16:0)	0,75	0,85	0,76	0,76	0,80	0,75	1,27	1,26	1,33
	Threonic acid	1,40	1,07	1,36	1,78	1,34	1,63	0,99	1,14	1,13
	Unknown lipid (28000473)	0,23	0,27	0,21	0,17	0,32	0,30	0,50	0,77	0,60

b)	Metabolite	MCP			MCPA			2,4-DP		
		m7	m14	m28	m7	m14	m28	m7	m14	m28
	5-Oxoproline	0,98	0,81	0,78	0,66	0,69	0,69	0,97	0,99	1,03
	Alanine	0,67	0,71	0,67	0,68	0,77	0,83	0,81	0,84	0,97
	Deoxyribonucleic acids, total	0,81	0,82	0,70	0,94	0,87	0,77	0,50	0,78	0,72
	Ethanolamine plasmalogen (C39:5)	0,52	0,56	0,50	0,57	0,67	0,60	0,69	0,29	0,60
	Heptadecanoic acid (C17:0)	0,52	0,57	0,44	0,53	0,70	0,54	0,60	0,59	0,49
	Isopalmitic acid (C16:0)	0,39	0,46	0,27	0,48	0,47	0,25	0,41	0,77	0,47
	Tyrosine	0,74	0,89	0,76	0,87	0,77	0,87	0,89	0,94	0,89
	Uracil	0,75	0,83	0,71	0,79	0,88	0,75	0,84	0,88	1,07
	Uric acid	0,72	0,79	0,71	0,76	0,85	0,61	1,23	0,99	1,52

liver and kidney weights were greater for the high dose females. Relative liver and kidney weights were greater for both high dose males and females. No lesions were noted in the nervous tissue. For the other animals, a decrease in fat storage and an increase in incidence and severity of cytoplasmic eosinophilia and granular cytoplasm in the liver was noted for the high dose males and females. No observed adverse effect level was 500 ppm.

In a second study ten Wistar rats/sex/group were treated in the diet with 0, 100, 500 or 2500 ppm of 2,4-DP for 3 months, corresponding to a test substance intake of 0, 7.2, 36.7, 193 mg/kg/day (males), 0, 8.3, 41.4 and 208 mg/kg/day (females).

The mean body weights for the males and females in the 2500 ppm treatment group were 8.1 and 8.4% less than those of the control, respectively, at the end of the treatment. Water consumption was markedly increased for both sexes in the high dose group. In the hematology evaluation, mean red blood cell count, mean hemoglobin concentration and mean hematocrit were lower than the controls for both the males and the females in the 2500 ppm group. In the clinical chemistry, the 2500 ppm group had higher mean alanine aminotransferase, alkaline phosphatase, urea, creatinine and total bilirubin values than those of the control. The mean globulin (both sexes), triglyceride (males) and cholesterol (males) concentrations for this group were lower than those of the control. The mean absolute liver weights for both sexes in the 2500 ppm group were greater than those of the control. The mean relative weights for the kidneys of the 500 and 2500 ppm males and the 2500 ppm females were greater than those of the control.

Peripheral fatty infiltration of the liver was lacking in the high dose animals. The no observed adverse effect level was 500 ppm.

7. Conclusion 90-day toxicity

The two 90-day studies demonstrate reduced body weight development at dose levels 2000/2500 ppm for males and 2500/3000 ppm for females. At these dose levels a reduction of red blood cells parameters is observed. Clinical-chemical investigations demonstrate an effect on the liver (increased alanine aminotransferase and alkaline phosphatase activity as well as reduced globulin, triglyceride and cholesterol values). Clinical chemistry and urinalysis also point to the kidney as a target organ, as evidenced by increased urea and creatinine values as well as reduced specific urine gravity and possibly an increased presence of erythrocytes and bacteria. Pathological investigations confirmed the liver and kidney as target organs based on increased organ weight and the absence of peripheral fatty infiltration of the liver. The NOAEL in both studies was 500 ppm.

7.1. MCP (reviewed by California Environmental Protection Agency, 1999)

MCP was admixed to the feed at concentrations of 0, 75, 500, 2500 (males only) or 3000 (females only) ppm (corresponding to 0, 5, 35, or 189 mg/kg/day, respectively, for males and 0, 6, 41, or 240 mg/kg/day, respectively, for females) and fed to 15 Wistar rats

Table 14

Differentially regulated metabolites in phenoxy test substances in female animals. Listed are all metabolites which were commonly regulated in two of the test substances (MCPP and 2,4-DP (a); MCPP and MCPA (b)), but differentially regulated in the third substance.

a)	Metabolite	MCPP			2,4-DP			MCPA		
		f7	f14	f28	f7	f14	f28	f7	f14	f28
16-Methylheptadecanoic acid	0,54	0,51	0,55	0,62	0,49	0,62	0,49	0,34	0,38	
3-Hydroxyindole	1,71	2,68	2,59	2,75	3,30	2,99	1,11	1,23	1,20	
3-Indoxylsulfate	1,85	1,68	3,48	2,11	3,32	3,55	1,11	1,49	0,77	
3-Methoxytyrosine	0,75	0,72	0,89	0,49	0,45	0,58	0,63	0,70	0,91	
Alanine	0,55	0,58	0,63	0,73	0,72	0,80	0,76	0,60	0,48	
Cholesterylester C20:4	0,34	0,27	0,35	0,22	0,21	0,24	0,36	0,41	0,32	
Cysteine	0,56	0,67	0,66	0,50	0,68	0,77	0,73	0,89	1,35	
Cystine	0,53	0,44	0,57	0,60	0,57	0,93	0,89	0,96	1,60	
Indole-3-acetic acid	0,31	0,29	0,32	0,53	0,48	0,29	0,52	0,95	1,06	
Lysophosphatidylcholine (C17:0)	0,80	0,55	0,59	0,56	0,60	0,63	0,91	0,45	0,41	
Lysophosphatidylethanolamine (C22:5)	1,36	1,63	1,67	1,31	1,40	1,39	1,26	1,43	1,26	
Metanephrene	0,63	0,62	0,36	0,49	0,57	0,57	0,78	1,25	1,12	
Myristic acid (C14:0)	1,26	1,64	1,82	2,14	1,61	1,40	0,97	0,87	0,98	
Palmitoleic acid (C16:1cis[9]1)	1,45	2,52	3,27	2,48	2,17	1,90	0,85	1,12	1,55	
Phosphate (inorganic and from organic phosphates)	0,78	0,72	0,75	0,76	0,83	0,90	0,88	1,03	1,14	
TAG (C16:1,C16,1) and TAG (C14:0,C18:2) (putative)	1,82	3,72	3,83	4,61	4,97	2,84	1,60	1,60	3,61	
TAG (C52:5 (H) or C50:2 (Na)) (putative)	4,98	4,60	4,36	17,26	9,09	3,81	4,64	3,06	3,63	
TAG (putative)	2,05	1,86	1,82	2,89	2,46	1,80	1,95	1,55	1,57	
Threonic acid	1,61	1,44	1,44	1,44	1,81	1,89	1,42	1,04	1,16	
Unknown lipid (68000033)	0,61	0,62	0,68	0,64	0,57	0,66	0,70	0,46	0,49	
Uracil	0,63	0,70	0,56	0,53	0,86	0,72	0,82	0,87	0,81	
Xylitol	2,25	1,48	1,77	2,29	1,31	1,47	1,20	0,73	0,68	

b)	Metabolite	MCPP			MCPA			2,4-DP		
		f7	f14	f28	f7	f14	f28	f7	f14	f28
1,5-Anhydrosorbitol	1,28	1,44	1,42	0,76	0,40	0,47	1,02	0,96	1,15	
3,4-Dihydroxyphenylacetic acid (DOPAC)	1,43	2,99	1,75	3,20	2,50	2,81	0,79	1,84	1,53	
Arachidonic acid (C20:1cis[5,8,11,14]4)	0,54	0,66	0,83	0,62	0,59	0,44	0,82	0,80	0,66	
Docosapentaenoic acid (C22:1cis[7,10,13,16,19]5)	0,65	0,55	0,54	0,54	0,28	0,22	1,44	0,67	0,64	
Normetanephrene	0,88	0,80	0,57	1,79	1,64	1,74	0,92	0,77	0,90	
Phosphatidylcholine (C16:0,C16:0)	1,17	1,44	1,40	1,52	1,80	1,78	1,43	1,14	1,26	
Phosphatidylcholine (C16:0,C22:6)	0,86	0,65	0,60	0,92	0,51	0,40	1,20	0,81	0,98	
Phosphatidylcholine (C18:0,C18:2)	1,08	1,20	1,34	1,13	1,65	1,49	1,30	1,01	0,99	
Phosphatidylcholine (C18:1,C18:2)	1,65	1,90	1,78	1,68	2,12	1,78	1,88	2,33	1,71	
Pseudouridine	1,01	1,37	1,18	1,06	1,25	1,26	0,87	1,21	1,06	
Stearic acid (C18:0)	0,61	0,73	0,81	0,63	0,64	0,59	0,95	0,89	0,81	
trans-4-Hydroxyproline	0,71	0,70	0,77	0,60	0,50	0,56	0,88	0,89	0,78	

per sex per dose continuously for a period of 3 months.

No animals died and no clinical signs were observed. Treatment-related decreased mean body weight, decreased mean food consumption, and increased mean water consumption were observed in males at 2500 ppm and in females at 3000 ppm. Treatment-related decreased mean red blood cell, hemoglobin, and hematocrit levels in males at 500 and 2500 ppm and in females at 3000 ppm were observed. Treatment-related increases in alkaline phosphatase in both sexes, alanine aminotransferase in females, urea in both sexes, and creatinine in males were observed at 2500 ppm. A treatment-related increase in transitional epithelial cells in the urine was observed in males at 2500 ppm. Treatment-related increases in mean relative liver weights in both sexes at the high dose level and in mean relative kidney weights in both

sexes at 500 ppm and the high dose level were observed. Macroscopic examination revealed treatment-related discoloration of the adrenal glands in both sexes at the high dose level. Microscopic examination revealed a dose-related decrease in fat storage in the liver in males at 500 and 2500 ppm and in females at 3000 ppm, and treatment-related bile duct proliferation, severe cytoplasmic eosinophilia of hepatocytes, hepatocytes with granular cytoplasm (moderate to severe), and lipid storage in the adrenal cortex were observed in both sexes at the high dose level. No treatment-related effects were observed during functional observation battery and motor activity assessments. No neurotoxic effects were observed at gross necropsy or microscopic examination. The NOEL was 75 ppm. The US-EPA considers the NOAEL to be 500 ppm.

8. Discussion

The metabolome evaluation of the source substances indicate liver and kidney as the target organs. The metabolome evaluation of the target substance provides the same information. The metabolome profile associated with the liver indicates a lipid reducing activity, comparable with the one induced by peroxisome proliferators. This is fully in line with investigations performed already decades ago (Vainio et al., 1983). The identification of the kidney as a target organ is particularly important because a hallmark of the phenoxy herbicides is the saturation of renal excretion and reabsorption causing a rise in compound blood levels associated with the onset of toxicity (Timchalk, 2004; van Ravenzwaay et al., 2004). The metabolome pattern observed for renal toxicity is that associated with inhibitors of weak organic acid excretion and with reference compounds such as probenecid (Wang et al., 2014).

The overall comparison of the metabolome data indicate that 2,4-DP is the best source substance. Using the information of the 90-day study of this compound, it would have been predicted that MCPP would have shown decreased food consumption and body weight gain at 2500 ppm. The target organs are the liver (weight increase and clinical-pathology changes), as well as the kidney (weight increase and clinical-pathology changes). The metabolome analysis did not predict effects on the red blood cells as observed in the 90-day studies. It should be noted, however, that in the 28 day study with MCPA at a dose level similar to the one used in the 90 day study, also no effects on red blood cells were noticed. Therefore, the lack of identification of this effect in the metabolome investigations may be related to the fact that the effect simply had not manifested itself yet. The type of red blood cell changes observed with the three phenoxy-herbicides after the 90-day treatment suggests that this is related to a reduction in bone marrow activity. An in depth analysis of the metabolome of the three compounds did reveal changes in a few metabolites which are consistently associated with anemia (reduced cytosine and deoxyribonucleic acids) in several patterns for anemia. Additionally, there was a weak match with a pattern indirectly associated with reduced red blood cell values. These changes, however, were not sufficient to call this a match. Based on the results of the 90-day study with 2,4-DP for comparison a moderate reduction of red-blood cell parameters would, however, be expected at 2500 ppm also for MCPP. The NOEL would have been expected to be below the value of 2,4-DP, i.e. < 500 ppm and more likely in the range of that of MCPA, i.e. at least 150 ppm.

From a qualitative point of view, these predictions are very similar to the results of the actual 90-day study in rats performed with the target substance (reduced food consumption and body weight gain, target organs: liver and kidney – weight increases with concomitant clinical-pathology changes, reduced red blood cells values). From a quantitative point of view the predicted NOEL of 150 ppm for MCPP is in the range of that of the actual study (NOEL 75 ppm, NOAEL below 500 ppm). Thus, the 90-day rat toxicity study of the target substance (MCPP) could have been waived and substituted by the 90-day results of 2,4-DP. The NOAEL of MCPP would have been correctly assessed as < 500 ppm, and using MCPA's 90 day study (second source compound) values as at least 150 ppm.

This case study was presented at ECHA's workshop on New Approach Methodologies in Regulatory Sciences held in Helsinki, 19–20 April 2016. An integral part of presenting a read-across case is the compliance with the RAAF (read across assessment framework, see http://echa.europa.eu/documents/10162/13628/raaf_en.pdf). For this case study, the respective tables were prepared and shown here as [supplementary material](#). One of the main points of discussion of this case study was the prediction of “negatives” for

endpoints. Acknowledging that the prediction of absence of an effect always is challenging, some points can be made. Importantly, conclusions are drawn from a multi-angel perspective. Firstly, we identify if there are matches between the fixed metabolite patterns established with reference compounds, grouped by particular toxicological effects (MoA), and the substance investigated. Currently, we established about 120 individual patterns to which such a comparison is made. Then we compare the overall metabolome profile of the substance investigated with the complete profiles of all other compounds and rank these according to best matches. Finally, we evaluate the strength of the total profile. A compound is considered “negative” if it does not have moderate – good matches with the fixed patterns, if the association of the overall profile with any of the other overall profiles is low (correlation coefficient < 0.5), and if the strength of the pattern is in the range of control values. A further indication of the sensitivity of metabolomics versus classical toxicology comes from a comparison between the two. We compared the outcome of classical toxicology (with respect to NOEL and LOEL) for > 100 compounds with that of metabolomics, according to the above mentioned criteria, and noted that the sensitivity of both analysis is generally quite similar (van Ravenzwaay et al., 2014).

The workshop participants accepted the presented case study as scientifically valid and as a good example how new technologies can help to substantiate claims of substantial similarity between chemicals. A number of issues were raised which merit to be presented here.

- (1) The presented source substances clearly lack one chemical, i.e. 2,4-dichlorophenoxy acetic acid (2,4-D). This compound was not included because the appropriate metabolome data were not available. It should however also be noted that 2,4-D is chemically “least” comparable with the target substance. Nevertheless, the toxicity of 2,4-D is not dissimilar to that of MCPP; i.e. dominated by liver and kidney toxicity, changes in red blood cell parameters (FAO, 1996).
- (2) It was assumed that all impurities of the source and target compounds were known and that their presence was not relevant for the final outcome of the toxicity assessment. Given that all compounds used for this case study are registered agrochemicals, with known impurities this was not considered a problem.
- (3) Transparency of the process how to derive conclusions from the metabolome analysis is key to understanding its outcome and to build trust.
- (4) A framework for data capture and storage in a GLP (like) fashion and a consistent way to interpret such data would also help to increase applicability of new technologies. The latter is indeed a general issue and currently a topic for which ECETOC is organizing a workshop. For the metabolomics work presented here data the rules for data interpretation have been described extensively in (van Ravenzwaay et al., 2015).
- (5) The sensitivity and ability of metabolomics to detect changes, and the ability to demonstrate the absence of an effect is the most important factor to rely on this technology for prediction of effects and determination of a no observed effect level. Since 2008 BASF has used this technology for early prediction of toxicological effects, and within the context of studies performed under REACH a comparison of prediction and real study outcome was possible. From the analysis of these data it can be concluded that metabolomics is as sensitive as a guideline toxicological study (van Ravenzwaay et al., 2014). The rate of correct prediction of effects from compounds with an unknown toxicological profile was 83%,

the correct rate of demonstrating absence of effects was 84% (manuscript in preparation).

'Omics technologies have the advantage that they can be easily combined with the standard guideline studies. In our case we have used the 28-day (OECD 407) guideline study to obtain 0.2 ml of plasma used for metabolome investigations. This procedure does not interfere with the outcome of the study and it is conceivable that other technologies, e.g. transcriptomics, could also be combined with regulatory OECD studies without compromising their validity. The combination of classical observations, including full pathology, and the mechanistic information provided by e.g. metabolomics, provides an ideal basis for comparison of compounds. Within the MetaMap[®]Tox system that we use we obtain the comparison of a selected compound with all other compounds (currently ca. 800) based on their metabolomic profile within a few seconds. For large numbers of chemicals rapid information on potential biological similarity is a great advantage to start a more thorough evaluation of toxicological similarities. The omics information, together with the evaluation of the classical toxicological parameters from the 28-day study, then forms the basis of a substantiated claim to waive the performance of the 90-day study for the selected compound, if the reference compound(s) are/is convincingly similar. The data-base used to prepare the case study is at this time only suitable for systemic toxicity (i.e. predicting the toxicity of compounds for short-term to subchronic administration periods. It is not suitable for reproduction toxicity as we have not gathered metabolome profiles for this end-point. It may be quite suitable to investigate maternal toxicity or effects on fertility in parental animals. Whether this technology could also be used to assess embryo-toxicity or effects on the next generation is an open question.

Read across is and will remain the most important tool to reduce animal testing while providing risk assessors with the necessary information to do their work. Ball et al. (2016) note that clear focal points of work to enhance the good use of the read across concept are amongst others, to identify best practices for using biological profiling/bioinformatics tools to support establishing similarity of source and target chemicals in read-across. Failure to derive some best practices for their use will likely lead to significant uncertainty into how to use these tools effectively and also create some mistrust in the data where they are not used appropriately. Here we demonstrate that data from new technologies can help to increase the quality of read across cases and should enhance the likelihood of providing successful cases to the regulatory authorities. It should be noted, however, that in the present study target and source substances were structurally very similar, as this was a prerequisite to serve as a case study within the RAAF context. The great upside potential of such new technologies could be that they do not necessarily have to rely on an estimate of chemical similarity, but rather provide a biological foundation for similarity. We have referred to this concept earlier as "going from QSAR to QBAR" (quantitative biological activity relationship) (van Ravenzwaay et al., 2012). Such a concept would significantly enlarge the possibilities for read-across cases, while reducing the risk of incorrect read across proposals because occasionally small changes in chemical structures can have profound toxicological consequences. Further case studies are needed to demonstrate that this potential can indeed come to fruition, ideally also using other 'omics technologies.

A further, and far more significant step towards the reduction of animal testing and provision of mechanistic information would be to perform metabolomics studies in vitro. This can indeed be done in vitro, and we have established a system for the identification of liver toxicity modes of action as well as cell energy metabolism

(Balcke et al., 2011; Bordag et al., 2016). It should be noted, however, that the experimental set-up and establishment of a robust and reproducible test system is time consuming. Only when this is achieved can the development of a database with reference compounds be started. This process then has to be repeated for at least all major organs investigated in regulatory toxicity studies. This is achievable, but requires stamina, patience and a lot of commitment.

9. Disclaimer

This case study has been designed to illustrate specific issues associated with read-across and stimulate discussion on the topic. It is not intended to be related to any currently ongoing regulatory discussions on this group of compounds. The background document has been prepared to facilitate the discussion at the Topical Scientific Workshop and does not necessarily represent ECHA's position.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.yrtph.2016.09.013>.

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.yrtph.2016.09.013>.

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