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

B. van Ravenzwaay*, S. Sperber, O. Duerr, E. Fabian, F. Faulhammer, H. Kamp, W. Mellert, V. Strauss
BASF SE, Ludwigshafen, Germany

* Corresponding author. Tel.: +49 621 605 64 19; fax: +49 621 605 81 34.
E-mail address: bennard.ravenzwaay@basf.com (B. van Ravenzwaay).

Abstract

The purpose of this paper is to provide a case study of how new technologies, such as metabolomics, can be used to address chemical grouping and read across from a biological perspective. To demonstrate this, we selected MCPP as the target substance and as source substances MCPA and 2,4-DP.

The 28-day metabolome evaluation of the source substances indicate the liver and the kidney as the target organs. The metabolome evaluation of the target substance provides the same information. 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 2,500 ppm. The target organs are the liver (weight increase and clinical-pathology changes), as well as the kidney (weight increase and clinical-pathology changes). A moderate reduction of red-blood cell parameters would also be expected at this dose level. 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 NOAEL of 150 ppm 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 could have been waived and substituted by the 90-day results of 2,4-DP. The NOAEL would have been correctly assessed as < 500 ppm, and using MCPA's values, as at least 150 ppm.

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.

Introduction

The purpose of this paper is to provide an example (case study) of how new technologies, such as metabolomics, can be used to address chemical grouping and read across from a biological perspective. To serve this aim, we chose to compare three phenoxy herbicide active ingredients. We selected MCPP (also referred to as Mecoprop or Mecoprop-p) to be used as the target substance and MCPA and 2,4-DP (also referred to as Dichlorprop or Dichlorprop-P) as source substances. The active ingredients MCPP and 2,4-DP are phenoxy propionic-acids and have a chiral centrum. In the past, these compounds were produced as racemic (50:50) mixtures of the two isomers. Since the 1990ies the production has been modified to only produce one isomer (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, it is not surprising that the toxicity of racemic mixture and single isomer was shown to be identical. The modern toxicological package for both compounds has been generated in the 1990ies and 2000s with the single isomer. The metabolome studies presented here, were also performed with the single isomer. It should also be noted, that there are more phenoxy herbicdes 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 the read across assessment framework (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.

Identity of the target substance

Mecoprop-P (MCPP)

CAS number: **93-65-2**

Chemical name: (RS)-2-(4-Chloro-2-methylphenoxy)propanoic acid

Appearance: solid

Solubility in water: 900 mg/L(20 °C)

Linear Formula: C10H11ClO3

Melting point/range: 94 to 95 °C

Structural formula:

Molecular Weight: 214.65 g/mol

Identity of the sources substances

MCPA

CAS number: **94-74-6**

Chemical name: 2-methyl-(4-chlorophenoxy) acetic acid

Appearance: solid

Solubility in water: 300 mg/l (25°C)

Linear Formula: ClC6H3(CH3)OCH2CO2H

Melting point/range: 115.4 to 116.8°C

Structural formula:

Molecular Weight: 200.62 g/mol

Dichlorprop-P (2,4-DP)

CAS number: **120-36-5**

Chemical name: 2-(2,4-dichlorophenoxy)propionic acid

Appearance: solid

Solubility in water: 720 mg/L at 20 °C (R-isomer)

Linear Formula: C9H8Cl2O3

Melting point/range: 116 to 120 °C

Structural formula:

Molecular Weight: 235.064 g/mol

Conclusion: the target substance and source substances are structurally similar. The target substance MCPP is a phenoxy-propionic acid, and as such comparable with phenoxy propionic 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 (Figure 1).

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

Figure 1 Tanimoto scores for MCPP, 2,4-DP and MCPA using the MACCS keys software.

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

Table 1 Summary of acute toxicity of the two source substances MCPP 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
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

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.

Absorption, Distribution, Metabolism & Excretion

MCPP (reviewed by California Environmental Protection Agency, 1999).

Male and female Wistar rats were treated orally with ^{14}C-MCPP. Five rats/sex were dosed with 5 (Groups A, B, D) or 100 mg/kg bw/day (Groups C and E). The rats in Group B received 14 doses of unlabeled MCPP at 5 mg/kg bw/day prior to being dosed with the radiolabeled material. In addition, 12 rats/sex were dosed with 5 mg/kg bw/day (Group F). In Groups A, B and C, urine and feces were collected for 7 days. Expired air was collected from two males in Group C. In Groups D and E, blood samples were collected for 7 days. In Group F, four animals/sex/time point were euthanized at 0.5, 3 and 6 hours after dosing.

Results: Absorption of the administered dose ranged from 82.92 to 100.47%. Excretion was predominantly via the urine with the percentage of the total dose recovered in the urine and cage wash ranging from 79.74 to 100.06%. In Group A, 95.29 and 92.29% of the administered dose was collected in the urine and the cage wash within the first 24 hours for males and females, respectively. Repeated dosing reduced the amount collected in the urine and the cage wash during the first 24 hours to 88.97 and 86.46% for males and females, respectively. Likewise, at the 100 mg/kg bw/day dosing level, the percentage collected in the urine and the cage wash during the first 24 hours was reduced to 61.18 and 56.78% for males and females, respectively.
The total radiolabel recovered in the feces ranged from 3.56 to 12.52% of the administered dose. No radiolabeled material was recovered in the expired air. Radiolabel in the tissues and organs 7 days after dosing was predominantly in the fat followed by the skin, adrenals, kidneys and liver. In the plasma pharmacokinetic analysis, T_{max} values were 1.8 and 2.7 hours for males and females, respectively in Group D and 4.2 hours in Group E. $T_{1/2}$ for elimination was 6.35 and 4.23 hours in Group

D and 7.89 and 7.79 hours in Group E for males and females, respectively. The only metabolite identified in the study was hydroxymethyl-MCPP. Overall, unaltered test material and the hydroxylated metabolite constituted 92.29 to 95.34% of the recovered radiolabel in the urine in the first 48 hours after dosing

MCPA (reviewed by JMPR 2012

Phenyl-labelled ^{14}C-MCPA was administered by gavage to groups of fasted male and female Sprague-Dawley rats. Following a single gavage ^{14}C-MCPA dose of 100 mg/kg bw (three rats of each sex), radioactivity was excreted predominantly in urine (approximately 95% and 86% of the administered dose in males and females, respectively), with relatively low levels eliminated in feces (6.2% and 5.6% in males and females, respectively). The majority of this radioactivity was eliminated within 48 hours after dosing (approximately 78% in urine and 5% in feces), with the remainder excreted from 48 to 192 hours. The urine of two rats of each sex that contained the highest levels of radioactivity was subjected to metabolite analysis using thin-layer chromatography (TLC). The main components were the parent molecule MCPA (approximately 71%) and one other undefined fraction (approximately 14%). Furthermore, three minor undefined fractions were detected (approximately 5%, 4% and 3%, respectively).
The excretion of radioactivity in bile, urine and feces was analyzed in three male bile duct–cannulated rats following a single gavage ^{14}C-MCPA dose of 100 mg/kg bw. Recovery of radioactivity was low (mean of 34.5%), which the authors attributed to the poor condition of the animals. The mean levels of radioactivity in 0- to 24-hour bile, urine and feces were 3.7%, 20.3% and 10.5%, respectively.
In a tissue distribution experiment, 14 rats of each sex received a single gavage dose of ^{14}C-MCPA at 100 mg/kg bw, with two rats of each sex sacrificed at various times for up to 192 hours for the analysis of radioactivity in plasma and tissues. The highest plasma and tissue concentrations of radioactivity were detected at the earliest sampling time (3 hours) and declined thereafter. At 3 and 6 hours after dosing, plasma contained the highest concentration of radioactivity. At later sampling times, the carcass, skin, fat and adrenals had higher levels of radioactivity compared with plasma. In a separate experiment, whole-body autoradiography of three rats of each sex that received a single gavage dose of ^{14}C-MCPA at 100 mg/kg bw and were analyzed at 6, 24 and 48 hours (one rat of each sex) confirmed this pattern of tissue distribution of radioactivity).

In a further tissue distribution experiment, 10 rats of each sex received up to 14 consecutive daily gavage doses of ^{14}C-MCPA at 1 mg/kg bw/day. Two rats of each sex were sacrificed on days 1, 5, 10, 14 and 18. The pattern of uptake, tissue distribution and elimination of radioactivity was similar to that observed in the preceding single-dose studies.

Phenyl-labelled ^{14}C-MCPA was administered to male and female Wistar rats (five of each sex per group) as a single gavage dose of 5 or 100 mg/kg bw. A separate group of rats (five of each sex per group) received 14 daily gavage doses of unlabeled MCPA at 5 mg/kg bw followed by a single gavage dose of ^{14}C-MCPA at 5 mg/kg bw. Rats were fasted for 4 hours prior to and after dosing. Urine and feces were collected at various times for up to 168 hours after administration of the radiolabeled dose. Rats were sacrificed at 96 hours (5 mg/kg bw dose) or 168 hours (100 mg/kg bw dose) after dosing, and various tissues were sampled. Radioactivity was analyzed in excreta and tissues. Metabolites of MCPA were analyzed in urine and feces by TLC, high-performance liquid chromatography and liquid chromatography–mass spectrometry. Samples were analyzed with and without acid and alkaline hydrolysis and deconjugation with β-glucuronidase and arylsulfatase. In a pharmacokinetics experiment, rats received a single gavage dose of ^{14}C-MCPA at 5 or 100 mg/kg bw, and blood was collected at various times for up to 168 hours and then analyzed for radioactivity. Standard pharmacokinetic parameters were calculated for total radioactivity. In an additional tissue distribution experiment, groups of 12 rats of each sex received a single gavage dose of ^{14}C-MCPA at 5 mg/kg bw. At 1, 3 and 6 hours after dosing, four rats of each sex were sacrificed, and various tissues were analyzed for radioactivity.

Results: No signs of toxicity were reported. Recovery of radioactivity was greater than 95%. Irrespective of the dose or dosing regimen, the kidneys were the main route of excretion and accounted for more than 93% of the administered dose in males and more than 88% of the administered dose in females (urine plus cage wash). The majority of excretion occurred within 24 hours of dosing. Based on the level of radioactivity in urine and the cage wash, gastrointestinal absorption is estimated to be at least 95%. The feces were a minor route of elimination, becoming more prominent in high-dosed females, for which fecal excretion attained 20% of the administered dose in two of five rats. During a pilot study involving two male rats from the repeated-dose group, no radioactivity was detected in expired air.

The distribution of radioactivity in selected tissues of rats sacrificed at the end of the excretion/mass balance study (96 or 168 hours) was analyzed and the highest level of radioactivity was detected in fat, followed by the skin and carcass. The level of residual radioactivity in the carcass was less than 0.8% of the administered dose. The slight retention of radioactivity in the ovaries and uterus of high-dose females was assumed by the study authors to be associated with visceral fat. Radioactivity was not detected in bone, brain, heart, lung, spleen, stomach contents or the thyroid.

2,4-DP (reviewed by California Environmental Protection Agency, 2002)

Male and female Wistar rats were dosed by oral gavage with ^{14}C-2,4-DP. For Groups A and D, 5 animals/sex were dosed with 5 mg/kg of the test material. In Group B, 5 animals/sex were dosed with 5 mg/kg of unlabeled 2,4-DP for 14 days, followed by a single dose of 5 mg/kg of the labeled test material. For Groups C and E, 5 animals/sex were treated with 100 mg/kg of the labeled test material. In Group F, 12 animals/sex were dosed with 5 mg/kg of the labeled material. For Groups A, B and C, urine was collected at 6, 12, 24, 48, 72, 96, 120, 144 and 168 hours after dosing with the radiolabeled material. Feces were collected from these animals at 24, 48, 72, 96, 120, 144 and 168 hours after dosing. Expired air was collected at 12, 24, 48, 72, 96, 120 and 168 hours after dosing from 2 animals in Group C. Each time excreta were collected, cages were rinsed and the cage wash was analyzed for radioactivity. Analysis of the radiolabel in various tissues and organs was performed at the end of 7 days. For Groups D and E, blood was collected from the caudal vein at 0 (pre-dose), 1.5, 3, 6, 9, 12, 24, 48, 72, 120 and 168 hours after dosing. For Group F, 4 animals/sex were euthanized at 1, 3, and 6 hours after dosing and tissues and organs were analyzed for the presence of radiolabeled material.

Results:
Excretion of the administered dose occurred largely in the urine during the first 24 hours after dosing. For both sexes, 94.3% or more of the radiolabel were recovered for Groups A and B by 24 hours and 62.2% or more was recovered in the urine. Another 11.5% or more were recovered in the cage wash. For the females in Group C, excretion of the radiolabel was similar with 91.5% of the administered dose excreted in the first 24 hours (65.2% in the urine, 6.6% in the feces and 19.8% recovered in the cage wash). In contrast, for the males in Group C, excretion was less rapid with 61% of the radiolabel recovered in the first 24 hours. By 48 hours, 95.6% of the dose was excreted with 77.5% in the urine, 11.0% in the feces and 7.0% in the cage wash.
Metabolism of the test material was minimal. Recovery of metabolites was largely limited to the urine with measurable quantities isolated in the first 6 hours after dosing. The parent compound represented 70% or greater of the recovered radiolabel for the males and 88.8% or greater for the females for these samples. Treatment with glucuronidase or sulphatase or with HCL or NaOH did not alter the profile appreciably. The test material was largely absorbed as indicated by the high percentage excreted in the urine.
Maximal plasma concentrations were achieved at 2 to 3 hours after dosing (5 mg/kg) or 4 to 5.5 hours after dosing (100 mg/kg). The mean $T_{1/2}$ values for elimination ranged from 4.4 to 16.5 hours. The elimination half-life, the mean $T_{1/2}$ values, ranged from 4.4 to 7.3 hours. Maximal tissue concentrations

were noted at 1 or 3 hours after dosing with the higher concentrations of the radiolabel in the heart, lung, liver, kidneys, thyroid and adrenals.

Conclusion ADME

Bioavailability for target and source substances is high (> 90% at low dose levels), to a somewhat lesser extent at higher dose levels. For all three 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. There is no elimination through the expired air. Fast elimination is reflected in relatively short 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. Overall the ADME properties of the target and sources substances are substantially similar.

28-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 profile following 28 days of compound administration is not very well defined (with the exception of MCPA).

Summary of toxicity of 28-day study rats with 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.

Results: No animal died during the conduct of the study. MCPA caused no clinical signs either during the study or in the functional observation battery and activity monitoring. Food consumption was significantly lower throughout the study in males fed 2000 ppm MCPA and in females at 2000 ppm during the last week of treatment. Body weights and body weight changes were significantly lower throughout the study in males fed 2000 ppm MCPA, and in females towards the end of the study. 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 fed MCPA at 2000 ppm. An increased number of degenerated transitional epithelial cells in three of five males receiving MCPA at 2000 ppm was observed.
At necropsy, males receiving MCPA at 2000 ppm 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 noted in the testes of three males.

Conclusion: 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. In addition, there were some effects

on red blood cell parameters. The testes may be an additional target organ, although the observed changes may be related to the decreased body weights in these young adult animals. A NOAEL was not determined in this study

Summary of toxicity of 28-day study rats with 2,4-DP (reviewed by California Environmental Protection Agency, 2002)

Ten Wistar rats/sex/group were fed in the diet 0, 100 or 500 ppm of either 2,4-DP racemate or of 2,4-DP D-form for 4 weeks (test substance intake 2,4-DP racemate: (M) 9 and 43 mg/kg/day, (F) 9 and 46 mg/kg/day, 2,4-DP D-form: (M) 9 and 42 mg/kg/day, (F) 9 and 45.0 mg/kg/day).

Results: 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.

Conclusion 28-day toxicity source substances: The NOEL for MCPA is slightly 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. The NOEL for 2,4-DP is above 42 mg/kg bw, a toxicity profile was not obtained. Based on this information it is not possible to draw conclusions with respect to the 28-day toxicity of the target substance in rats.

Summary of toxicity of 28 day study rats with MCPP (Reviewed by European commission 2012)

Wistar rats were fed a diet containing 0, 50 and 400 ppm MCPP. The 28-day study 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 this was considered to be 400 ppm, while the NOEL in this study was 50 ppm (equal to 4 (males) and to 5 (females) mg/kg bw/day).

Metabolomics

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). 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.,* 2014; Mattes et al., 2013; 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 a OECD 407 guideline design with two dose levels.

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 screen analysis. 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.

<u>Metabolome Data Evaluation</u>

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 2014). We have set a threshold for selectivity in such a way, that compounds which matche at least with 75% of the metabolite regulations in the established patterns fit to the MOA (> 75% = weak match; > 90 % = match, see tables 2-6). The metabolome 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 metabolome 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 metabolic 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 %; equivocal findings: approx. 50-75%; mismatches: < 50%). Furthermore, the quality and importance of the metabolite changes for a certain toxicological mode of action is considered for this evaluation. For example, metabolites which are based on perturbances of specific biochemical pathways, and which can be connected to the toxicity 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.

2. Profile Comparison

The profile comparison is performed on the basis of the entire metabolome profile of a target compound, against all other metabolome profiles available in the data base 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 data base 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 data base 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 130000 r-values/stratum.

Based on these analysis, 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.

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. Expert judgement

Final conclusions are drawn based on a multi-step procedure. First 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.

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 organisms. 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 not always easy to determine. It should also be noted that the plasma based metabolomics as presented here is not a high throughput technology. Building our data base 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 data base 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 data base 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 analysed.

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 analysed. 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).

Results of the Metabolome Analysis

<u>Pattern Ranking</u>

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 2.

Table 2 Pattern ranking of MCPA demonstrated the following matches / weak matches with pre-defined patterns in the data base.

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

(dark green)	Match
(light green)	Weak match

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.

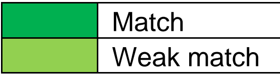
Evaluation of 2,4-DP: 2,4-DP was administered for 28 days to Wistar rats at dose levels of 1000 and 2500 ppm. Blood samples were taken at day 7, 14 and 28 for metabolome analysis.

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 3.

Table 3 Pattern ranking of 2,4-DP demonstrated the following matches / weak matches with pre-defined patterns in the data base.

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



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.

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 MCCP 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 4.

Table 4 Pattern ranking of MCPP demonstrated the following matches / weak matches with pre-defined patterns in the data base.

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	Weak match
		Liver oxidative stress	Weak match
		Liver PPAR alpha agonist	Weak match
	Female	Liver peroxisome proliferation	Match
		Kidney inhibition weak acids	Match
1000 ppm	Male	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

(dark green)	Match
(light green)	Weak match

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 higer 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.

Combined metabolome evaluation

All three compounds showed a clear effect on the liver, matching patterns for liver peroxisome proliferation and fibrate as well as phthalate induced liver toxicity, the MOA of these compounds being related to a lipid reducing effect, based on PPAR-alpha induction and subsequent peroxisome proliferation (table 5). 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.

Table 5 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	[Match]	[Match]	[Match]
Liver fibrate phthalate and phenoxy	[Match]	[Match]	[Match]
Reduced feed consumption	-	[Match]	[Match]
Kidney inhibition weak org. acids	[Weak match]	[Match]	[Weak match]
Phthalates long chain	[Weak match]	[Match]	[Match]
Liver PPAR alpha agonist	[Weak match]	[Weak match]	[Weak match]
Liver oxidative stress	[Weak match]	-	[Weak match]

Colour	Legend
(dark green)	Match
(light green)	Weak match

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 6).

Table 6 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

1. <u>Profile comparison</u>

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 MCPP 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 figure 2.

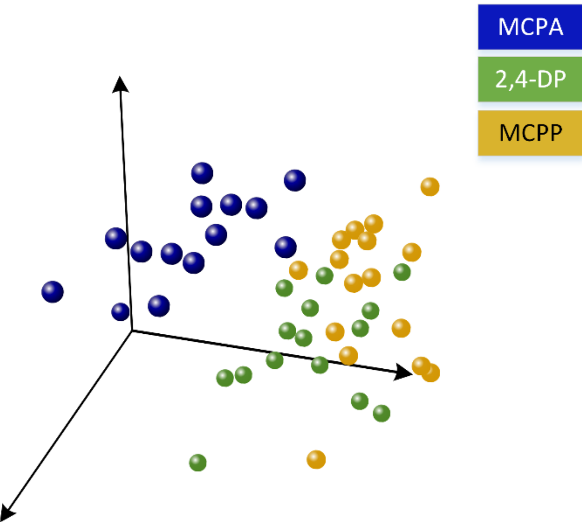


Figure 2 Principle component analysis of the metabolome of MCPA, 2,4-DP and MCPP. Overall profiles are similar, however, subtle compound differences can be differentiated. The metabolic profile produced by MCPP is more similar to the one produced by 2,4-DP than to the metabolic changes induced by MCPA.

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. (1) 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 7). This calculation was also performed for the commonly, significantly changed metabolites when a lower dose (1000 ppm) was administered.

Table 7 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

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:

$$P = (p_1, p_2, \ldots, p_n), \;\; p_x \neq na \qquad\qquad S_P = \left\lfloor \frac{\sum_{k=1}^{n} |p_k|}{n} \right\rfloor$$

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 8.

Table 8 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

The results of the evaluation of the overall profile strength indicate that MCPP and MCPA have a similar 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).

3. <u>Biochemical pathways</u>

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 35-43% 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 9). 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 dihomo-gamma-linolenic acid is up-regulated in males and females (table 9 and table 10). 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.

***Table 9** 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

Table 10 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

In table 11 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 12.

Table 11 *Differentially regulated metabolites in phenoxy test substances in male 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		
	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	MCPP			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

Table 12 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
Metanephrine	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:cis[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:cis[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:cis[7,10,13,16,19]5)	0,65	0,55	0,54	0,54	0,28	0,22	1,44	0,67	0,64
Normetanephrine	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

The fact that there are more metabolites commonly regulated between MCPP and 2,-DP than between MCPP and MCPA is further evidence indicating that 2,4-DP is more suitable as a sources substance than MCPA for read across to the target substance MCPP.

90 day toxicity studies in rats

MCPA (reviewed by JMPR 2012)

In a combined subchronic toxicity and neurotoxicity study, 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).

Results: At the highest dose, a significant decrease in body weight was observed in both sexes beginning on day 7 of the administration period and continuing until the end 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 amino transferase, alkaline phosphatase and aspartate aminotransferase) were observed. Histopathology demonstrated alterations of hepatocytes, characterized by cytoplasmic eosinophilia and granular cytoplasm in the liver. 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. No significant treatment-related changes were seen at the two lowest doses. The NOAEL was at 500 ppm.

MCPA was administered to 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).

Results: 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.

Conclusion: 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 a 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 suggest the NOAEL to be at 150 ppm .

2,4-DP (Reviewed by California Environmental Protection Agency, 2002)

Fifteen Wistar rats/sex/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, 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.

Results: 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 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 derived from the animals receiving perfusion fixation. 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.

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).

Results: 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. No observed adverse effect level was 500 ppm.

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 the 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.

MCPP (Reviewed by California Environmental Protection Agency, 1999)

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

Results: No animals died. 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 mean alkaline phosphatase in both sexes, in mean alanine aminotransferase in females, in mean urea in both sexes, and in mean 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.

Discussion

The metabolome evaluation of the source substances indicate the liver and the kidney as the target organs. The metabolome evaluation of the target substance provides the same information. 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 2,500 ppm. The target organs are the liver (weight increase and clinical-pathology changes), as well as the kidney (weight increase and clinical-pathology changes). A moderate reduction of red-blood cell parameters would also be expected at this dose level. 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 NOAEL of 150 ppm 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 could have been waived and substituted by the 90-day results of 2,4-DP. The NOAEL would have been correctly assessed as < 500 ppm, and using MCPA's values as at least 150 ppm.

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