Temporal trends of 4-Hydroxychlorothalonil in maternal serum samples, 1997-2015

ELIN WÄLLSTEDT 2022
MVEM12 DEGREE PROJECT FOR MASTER´S DEGREE 30 HP
ENVIRONMENTAL SCIENCE | LUND UNIVERSITY

Temporal trends of 4-Hydroxychlorothalonil in maternal serum samples, 1997-2015

A statistic fungicide study in Scania Sweden

Elin Wällstedt

2022

LUNDS UNIVERSITET

Elin Wällstedt

MVEM12 Degree project for Master's degree 30 hp, Lund university

Supervisor: Annette Krais, Occupational- and Environmental medicine south (Arbets- och Miljömedicin [AMM] Syd), Applied mas-spectrometry, Lund university

CEC - Centre for Environmental and Climate Science
Lund university

Lund 2022

Abstract

Agricultural fungicides are extensively used for infestation control, resulting in residues of these compounds in food. Human exposure to environmental contaminants might occur via different routes, such as inhalation, skin contact or ingestion. Exposure to several pesticides has been associated with adverse human health outcomes. Our aim was to assess the stability as well as temporal trends of a biomarker for the fungicide Chlorothalonil (CHT) in human serum. CHT has been classified a probable human carcinogen that has shown toxicity in the environment, fish, and mice. Samples had been collected from pregnant women (n = 1809 in Scania, Sweden, from 1997 to 2015. The concentration of the biomarker 4-hydroxychlorothalonil (4-OH-CHT) was analysed by Occupational- and Environmental medicine south (OEM south) in human serum using LC–MS/MS. The purpose of this study, to evaluate temporal trends in biomarker concentrations (log-transformed) using linear regression was carried out. A literature search was used to gather information about CHT. The biomarker of chlorothalonil (4-OH-CHT), was detected in 100% of the population in all sampling years. The biomarker´s median concentration was 4,1 ng/mL. No temporal trends were found for 4-OH-CHT (This means no annual change, indicated by β, = 0,0% change/year in Table 6). This is to our best knowledge the first study evaluating this factor in human samples. Further studies are needed to evaluate possible risks for offspring and mothers.

Populärvetenskaplig sammanfattning

Trender av svampbekämpningsmedlet Klorotalonil i gravidas blod över en 18årsperiod

Bekämpningsmedlet Klorotalonil (CHT) har funnits länge och använts på en mängd olika typer av grönsaker, vilket är förbjudet i Sverige och även Europa idag. Ändå hittades det nyligen i gravida skånska kvinnor och har fortsatt upptäckts i gravida kvinnors blod kontinuerligt under de senaste 18 åren. Sannolikt finns det i oss alla. Men hur hamnade det där? Svaret på denna fråga och frågor som denna är inte kända för forskningen. Lite forskning på möss, hundar med flera djur har utförts och det pekar på att CHT kan vara cancerogent för människor även om det är svårt att säga något exakt utifrån hur djur reagerar.

Att ställa upp halterna för alla 18 år i en lång rad gav ett rätt jämt resultat. Det skulle kunna betyda att vi var ungefär lika utsatta för det här bekämpningsmedlet för sju år sedan som för 25 år sedan, men dessa antaganden behöver undersökas i vidare studier på stabiliteten. Ingen annan har någonsin gjort en sådan tidsserie vare sig för ämnet i natur, djur eller människor, såvitt vi vet.

Vidare studier skulle kunna fortsätta arbetet med att undersöka till exempel om vi i Sverige har höga eller låga halter i blodet i förhållande till andra länder, om de som bor nära odlingar där ämnet används (utanför Europa) har högre halter, eller om de som äter en viss typ av grödor har högre halter. Men, även vad som kan påverka hur mycket som tas upp i ens kropp.

Viktigast av allt är dock att undersöka huruvida det alls finns koppling till någon hälsofara orsakad av det här ämnet. Löper barnen till dessa mödrar högre risk att ligga inom spektret för någon specifik diagnos exempelvis. Kan den diagnosen härledas kemiskt till något ämnet påverkat i barnets/mammans kropp? Går det att med tillräcklig säkerhet säga att det här ämnet borde begränsas? Det är det vi försöker sätta förutsättningarna för att motivera, utifall att det skulle visa sig vara relevant för att skydda människors hälsa. Förebyggande arbete när det är som färskast är vad du skulle kunna fortsätta läsa om här.

Keywords Biomonitoring ● Human exposure ● Fungicide ● Study on pregnant women ● Chlorothalonil ● CHT ● 4-hydroxy-chlorothalonil ● 4-OH-CHT

Table of contents

Introduction

Fungicides are a broad group of chemicals extensively used over the last century to control pest infestations. Chlorothalonil (CHT) is a broad-spectrum organochloride fungicide that is also used in paints. It was first developed in the US in 1966 (Kwon & Armbrust, 2006), and has since then been applied when cultivating vegetables, including, peanuts, cabbage and tomatoes (Battaglin et al., 2008; Hou et al., 2016; Kwon & Armbrust, 2006).

The mechanism of action of CHT in mold/fungi is inhibiting thiol enzymes and sulfhydryl groups. CHT causes cell death by inhibiting the enzyme function of glutathione (GSH) (Kwon & Armbrust, 2006; Tillman et al, 1973).

The half-life of chlorothalonil varies in different types of plants, and in different climate zones (Hou et al, 2016). The dissipation half-life of chlorothalonil in greenhouse-grown vegetables was estimated to 5.3d (cucumber), 7.3d (pepper), 11.5 d (cherry tomato), 2.2 d (banana) and 1.8-3.2 d (spinach) (Chaves et al., 2007; Lin et al., 2019; Ramirez et al., 2019; Valverde-Garcia et al., 1993).

Chlorothalonil has been detected in streams and rainfall (Armbrust, 2001; Battaglin et al., 2008; Sakai, 2002). It can be degraded in water under sunlight exposure, with half-lives ranging from one to 48 hours depending on the water composition and light source (Sakkas, 2002).

CHT is moderately persistent in soil and is primarily degraded by a variety of microorganisms to 4-hydroxychlorotalonil (4-OH-CHT) (P.Raman, 2014; Rouchaud et al., 1988; Shi et al., 2011). 4-OH-CHT showed greater stability and wider distribution compared to other biotransformation products (Kwon & Armbrust, 2006). Therefore, 4-OH-CHT is considered as the major threat resulting from the CHT application in agriculture and recreational sports (Gamble et al., 2001; Xu et al., 2020). It is more toxic to birds but less toxic to fish and aquatic invertebrates than chlorothalonil (U.S. Environmental Protection Agency, 1999).

The U.S. Environmental Protection Agency classifies chlorothalonil as a group B2 (probable human) carcinogen with very high toxicity to fish and aquatic invertebrates (U.S. Environmental Protection Agency, 1999) but low toxicity to birds and mammals (Battaglin et al., 2008). Oral toxicity tests of chlorothalonil in mice have showed a significant embryo lethality and a reduction in live fetuses (Farag

et al., 2006). 4-OH-CHT has demonstrated toxic effects on aquatic organisms, causing oxidative stress and genetic toxicity in fish (Lopes et al., 2020). At the concentration of 50 μg/L, the fatality rate of 4-OH-CHT to zebrafish has been shown to be 2.6 times higher than that of chlorothalonil, and 4-hydroxychlorothalonil has also showed stronger endocrine disrupting effect in zebrafish than its parent chlorothalonil (Quan et al., 2016).

Despite the fact that 4-OH-CHT has been released into the environment and the direct contact with 4-OH-CHT that may happen during the agricultural and recreational activities, there is nearly no record on the toxicity of 4-OH-CHT to human health (Xu et al., 2020).

According to a review performed by United Nations Environment Programme (UNEP), World Health Organisation (WHO) and International Programme on Chemical Safety (IPCS) the toxicological data for chlorothalonil revealed that the most important studies for human risk estimation was long-term effect of chlorothalonil in dogs since this was the most representative species for humans. The conclusions stated that the No-observed-effect-level (NOEL) of 120 mg/kg in the diet in the 2-year study on dogs, was equivalent to 3 mg/kg body weight per day, and therefore should be used for the purpose of human risk estimation (UNEP, WHO & IPCS) Chlorothalonil has an acceptable daily intake and a reference dose value of 0.03 and 0.015 mg kg−1 day−1, respectively (Raman 2005).

The primary routes of exposure to CHT occur via ingestion, inhalation, dermal, and ocular contact (P.Raman, 2014). Chlorothalonil gets rapidly absorbed when ingested and inhaled. Liver is the primary site of metabolism of chlorothalonil via conjugation with glutathione. It is a hepato-, nephro-, neuro-, and reproductive toxin with carcinogenic potential (P.Raman, 2014).

Occupational exposure to chlorothalonil may occur through inhalation of dusts or dermal contact with this compound at workplaces where it is produced or being used as a pesticide. The greatest potential for dermal and inhalation exposure to chlorothalonil is expected for pesticide applicators and farm workers who have frequent contact with products containing this compound (Raman, 2014). Contact dermatitis has been reported for personnel working in chlorothalonil manufacturing, in farmers and in horticultural workers (UNEP, WHO & IPCS).

At the end of 1990s the approval expired for the last product where chlorothalonil was included as active substance in Sweden (Kemikalieinspektionen [KEMI], 2021). In 2016 the European Parliament and Council adopted a regulation which regards maximum residue levels for chlorothalonil in products as fruits and vegetables (Regulation 2016/67). In April 2019, the European Union did not renew the approval of the active substance chlorothalonil (Regulation 2019/677).

Temporal time trend studies of exposure (also called Biomonitoring) can be used to surveillance exposure levels over several years and is an important element of exposure assessment (Ganzleben et al., 2017; Jakubowski et al., 2005; Yusa et al., 2015). Concentrations of chemical substances in human biological samples reflect the total environmental exposure from our surroundings. Currently used pesticides generally have short biological half-lives and are rapidly excreted in urine, even though certain substances sometimes residues in other parts of the body. Pourchet et al., for example were able to detect and identify 4-hydroxychlorothalonil (4-OH-CHT) in human milk with Liquid Chromatography (LC) and Gas Chromatography (GC) in 2021. However, so far scarcely any studies on pesticide exposure and time trends thereof have been made (Hendryx & Luo, 2018).

The objective of this study was to assess the CHT-biomarker 4-hydroxychlorotalonil (4-OH-CHT) and its potential temporal trends (1997–2015) in maternal serum. The sample serum originated from pregnant women in the general Scanian population (in the southernmost part of Sweden). The fungicide was selected mainly because it has been recently detected in untargeted screening of human blood (Plassmann, 2018). Furthermore, it is frequently used globally and has been detected in food products sold in Sweden (Livsmedelsverket, 2014). The compound furthermore was included because it has rarely been monitored in humans. No research has so far examined the potential for bioaccumulation of CHT or its metabolites in humans.

Aim and research questions

The **main aim** is to visualize possible time trends of the substance 4-hydroxychlorothalonil in human blood. The results from the time trend in this study and the understanding of these results may contribute to, or possibly motivate, further studies on human exposure. Descriptive data considering toxicity mechanisms of CHT and possible health effects will be described using previous studies. Regarding the biomarker 4-OH-CHT, data analysis will be carried out and presented regarding possible temporal trends. These results will be visualized by a box-plot-graph.

Chlorothalonil

- **What is chlorothalonil (CHT), and what is it used for?**

Toxicity mechanisms

- **What is the toxicity mechanism of CHT?**
- **What health effects are seen in animals?**
- **What are the health effects in humans?**

Metabolites/Biomarkers

- **How can we measure metabolites with biomonitoring?**
- **Are there any temporal trends in maternal serum concentrations 1997-2015?**
- **Did the concentrations differ between the seasons?**

Method

Selection of scientific studies included in the literature review

This study is intended to examine human exposure of CHT. When studies made on animal-exposure of CHT are mentioned in this study there was a lack of studies on human exposure of CHT in science. The studies should include effects on human exposure and health. Chlorothalonil's effects on ecosystems, flora and fauna will not be included in this study nor the stability of this substance in different media such as water, air or likewise. Different exposure pathways will not be examined nor if there is a particular one that is more likely than the others. No questionnaires will be sent out to examine factors amongst human exposure.

The geographical limitations will be minimal as the number of studies within the subject are sparse. Since our samples originate from Swedish participants, studies from similar conditions as these will be prioritized if available. No limit for publication date will be set as findings dated back to the 1970's has shown relevant. Articles written in either Swedish or English can be included. Figures and tables from articles are prioritized to make the text as educational and accessible to the reader as possible.

Data collection

A literature search combined with data analysis will compose the method chosen. This is needed to give a thorough description of the background of the potential time trends that will be produced. The background material will be based on peer-reviewed scientific articles collected from the database Web of Science (WoS) or PubMed. Nevertheless, empirical material will come from the collected maternal serum samples (1997-2015) and the statistical analyses composed by using SPSS. AMM provide assistance from statisticians with the opportunity to assist the study for a longer period with expertise in SPSS analyses.

The literature search process began with a search for Chlorothalonil in WoS to provide an overview of the subject's scope in science (709 hits on the title). Keywords were added and combined until relevant titles and abstracts could be identified and picked out. The snowball method and search in sources of the found material are planned to occur as more material is requested. This resulted in 28 referred articles in this study amongst other sources of information. Table 1 shows the used search phrases, and the article selection is presented in Table 1.

Table 1. Terms used in the research process for scientific articles.

Block	Terms
Substance	chlorothalonil* OR 4-hydroxychlorothalonil OR ”chlorothalonil metabolite” OR chlorothalonil-4-hydroxy OR 4-OH- chlorothalonil OR 4-OH-CHT
Metabolization	stability OR duration OR metabolization* OR metabolis* OR metabol* OR stable OR excretion OR excreat* OR bioaccumulat* OR accumulat* OR store*
Population	“pregnant women*” OR “in Sweden” OR Sweden “human exposure” OR “in humans” OR pregnant* OR human
Biological matrix	”human samples” OR blood OR urine OR serum OR “blood sample” OR “urine sample” OR biological matrices OR biological samples OR ”breast milk”

Analytical method of 4-OH-CHT in serum

Chemicals, reagents, and materials

The analytical standard solution 4-hydroxychlorothalonil (4-OH-CHT) was purchased from Dr Ehrenstorfer (Augsburg, Germany). Acetonitrile and Methanol (Supelco®, LiChrosolv®, hypergrade for LC-MS), and ammonium acetate (EMSURE® ACS Reag. Ph Eur, for analysis) were from Merck (Darmstadt, Germany). β-Glucoronidase from E. coli K12 was from Roche Diagnostics (Mannheim, Germany). Water was from a Milli-Q® Integral 5 system (Millipore, Billerica, MA, USA). 96-well carrier plate with 1.5 ml flat bottom glass inserts, and a 96-plug solid sealing mat (Molded Flat Mat PTFE/Silicone White Liner); were all from the Multi-Tier™ MTP System Topas (J.G Finneran Associates, Vineland, NJ, USA). 96-well carrier plate (Deep, PP, 1.3 ml) with 0.5 ml conical glass inserts, and a 96-plug injectable sealing mat (Blue CapMat with Pre-Cut PTFE/Silicone Septa); were all from the WHEATON® MicroLiter Plate Sampling System™ (DWK Life Sciences, Millville, NJ, USA).

Instrumentation

Quantitative analysis was conducted using triple quadrupole linear ion trap mass spectrometers equipped with TurboIonSpray sources (QTRAP® 5500+, AB Sciex, Framingham, MA, USA) coupled to a liquid chromatography system (UFLCXR, Shimadzu Corporation, Kyoto, Japan; LC/MS/MS). Nitrogen was used as nebulizer, auxiliary, curtain, and collision gas. The MS analyses were carried out using selected reaction monitoring (SRM) in negative ion mode. The SRM conditions are shown in Table 2. All data acquisition was performed using Analyst 1.7.2 software and data processing was performed using Multiquant 3.0.1 (AB Sciex, Framingham, MA, USA).

Calibration standards, chemical blanks, and quality control samples

Standard solutions were prepared by further dilution of diluted reference standards in acetonitrile at a concentration of 400, 200, 100, 20, 10, 2 and 0 ng/ml. For the calibration standards, serum matrix (human serum from healthy volunteers) was used and prepared in the same way as the samples, except for the addition of 25 µl diluted standard solutions (i.e. after digestion step described in sample preparation procedure). One prepared reference sample was used for quality control (QC1) by pooling samples from individuals from our laboratory. The QC sample, four chemical blanks (water), and calibration standards were included in each sample batch (96-well plate). No internal standard (IS) was available for 4-OH-CHT. The mean value of 4-OH-CHT in the control sample QC1 was 6.75 µg/L with a coefficient of variation (CV) of only 7.25%.

Sample preparation of serum samples

Samples were stored at -20°C until analysis. The thawed and homogenized samples were prepared in 96-well carrier plates with 1.5 ml flat bottom glass vials. Aliquots of 100 µl serum sample were added with 10 µl β-glucuronidase and 10 µl 1M ammonium acetate buffer (pH 6.5). The 96-well plate was covered with a sealing mat and samples were digested and mixed at 37°C for 90 min. Thereafter, 25 µl acetonitrile was added to each sample, except for calibration curve matrix samples where 25 µl of the diluted standard solution were added instead. To precipitate the proteins, 200 µl of acetonitrile was added to all samples, the 96-well plate sealed again, followed by vigorous shaking for 30 min. The samples were thereafter centrifuged at 2600g for 10 min. The supernatant (200 µl) was transferred to a new 96-well plate carrier with 0.5 ml conical glass vials for analysis, covered with an injectable sealing mat, and again centrifuged at 3000g for 10 min before analysis.

Analysis of 4-OH-CHT

An aliquot of 5µl of the supernatant was analysed on the LC/MS/MS. A Genesis Lightn C18 column, 4 µm, 50 x 2.1 mm (avantor, VWR International, Lutterworth, UK) was used for the analysis before the injector to filter the mobile phases from contaminating substances. The analytical column was a Gemini NX-C18, 110Å, 3 µm, 100 x 2.0 mm (Phenomenex, Torrance, CA, USA). The mobile phases were A) 5mM ammonium acetate in water and B) methanol. The flow rate was 0.6 mL/min, the column was maintained at 55°C and the total analytical run-time was 8.2 min. The analysis was performed in negative ionisation mode, the DP was -80V, the ion source temperature was set at 600°C and the ion spray voltage was -4500V. Table 2 shows the technical details, such as the quantifier ions and qualifier ions) Q1-Q3) as well as, dwell times, collision energies (CE) and declustering potentials (DP) for 4-OH-CHT.

Table 2. SRM conditions and fragments of 4-OH-C.

ID	Q1 (Da)	Q3 (Da)	Dwell time (msec)	CE (V)	DP (V)
4-OH-CHT (1)	246.2	35.0	8.0	-68	-80
4-OH-CHT (2)	245.0	35.0	8.0	-68	-80
4-OH-CHT (3)	**245.0**	**175.0**	**8.0**	**-38**	**-80**
4-OH-CHT (4)	245.0	210.0	8.0	-34	-80
4-OH-CHT (5)	245.0	182.0	8.0	-40	-80

SPSS-analysis

IBM SPSS (version 27.0) was the software of choice used for the statistical analysis. 100% of the serum concentrations lied above the limit of detection. In this dataset the continuous (Numeric) variable was the concentration of 4-OH-CHT (ng/mL). The concentration of 4-OH-CHT did not follow a normal distribution according to Kolmogorov-Smirnov Test for Normality. To improve the normality the data was Log-transformed. Still a Kolmogorov-Smirnov Test for Normality didn't approve of normality. The categorical variable for time was Date. It represented each samples report-date (Sampling occurred 5-7 weeks before the report-date). Preferably Date would have been collection date, date of sampling. Unfortunately, only report-date is available and therefore only year was extracted to the continuous variables that were used and reported here.

Since the data was not normally distributed the descriptive statistics were done through nonparametric statistics. For each sampling year *n*, median and P25-P75 were described. To explore and visualize possible trends over time boxplots of log-transformed biomarker concentrations were plotted against calendar year. Possible seasonal and/or monthly trends were examined in the same way but plotted against non-transformed 4-OH-CHT values. The seasons were categorized as "Winter" – December, January, February, "Spring" – Mars, April, May, "Summer" – June, July, August and "Autumn" as September, October and November. Lastly, linear regressions with all observations included were performed. A R^2 (coefficient of determination) was included for the regression model, with the purpose of indicating what amount of the variance in concentration was explained by other factors than calendar year. For the non-parametric data a non-parametric test of the median values was made to expose possible significant differences in seasonal exposure.

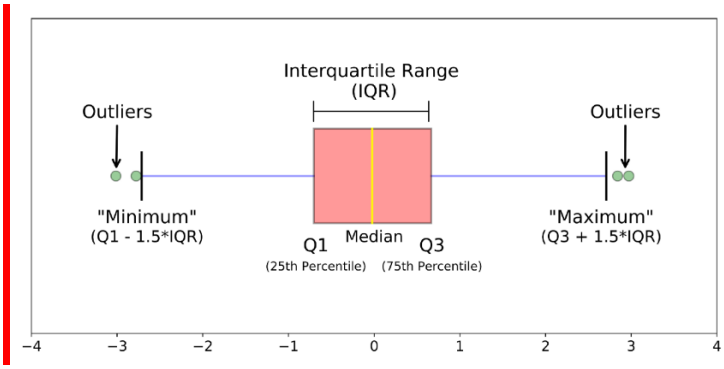

Figure 1. Understanding boxplots. Extreme outliers are data points that are more extreme than Q1 - 3 * IQR or Q3 + 3 * IQR (compared to mild/normal outliers shown as green dots in this illustration). Extreme outliers are marked with an asterisk (*) on the boxplot. *Sourse:* Galarnyk 2018.

Ethical reflection

This study mainly concerns human samples for the preventive purpose of the health of future generations. In the best-case scenario, there is clear reliable research that 4-OH-CHT is a suitable biomarker over time that does not interact with the body in a harmful way or accumulates or can be transmitted to a child from the mother. However, a stable biomarker could be used to observe possible time trends, at best showing a decrease in exposure to the fungicide chlorothalonil in Swedish pregnant women since the introduction of the ban in 1990. In this way, it might confirm that the ban had the intended effect.

Ethical approval has been granted (DNR 2015/221 - environmental toxins as risk factors for autism)

Results

The analytical method was validated and performed well with a limit of detection (LOD) of 0.1 ng/ml. The levels ranged from 0.16 to 38 ng/ml serum with a median level of 4.1 ng/ml. The biomarker 4-OH-CHT was found in serum in concentrations above LOD in 100% of the population in all sampling years. A boxplot of 4-OH-CHT (log transformed) concentrations versus sampling year is shown in Fig. 2. Descriptive statistics (i.e., *n*, median and P25-P75) are listed in Table 5 for each sampling year. After log transformation the 4-OH-CHT-concentrations met the assumptions for linearity, and could be evaluated using linear regression, shown in Fig. 2 and Table 6. No temporal trend was observed for the biomarker in the regression model, Fig. 2.

Table 5. Descriptive statistics of 4-OH CHT in blood sample concentrations.

Sampling year	***N***	**Median (ng/mL)**	**P25-P75**
1997	5	3.68	2.66-3.79
1998	31	3.50	2.09-4.43
1999	38	3.66	2.39-4.60
2000	40	3.19	1.99-4.60
2001	77	3.51	2.64-4.86
2002	84	3.85	3.12-4.57
2003	145	4.30	3.19-5.68
2004	111	4.49	3.21-5.75
2005	137	4.62	3.29-6.73
2006	151	4.52	3.31-6.49
2007	154	5.15	3.86-6.81
2008	189	4.76	2.81-7.35
2009	189	3.62	2.37-5.37
2010	160	3.70	2.53-5.29
2011	142	3.40	2.50-5.11
2012	88	3.71	2.65-5.09
2013	62	4.29	2.88-5.51
2014	5	4.77	2.52-7.31
2015	2	5.34	**2.90-5.34 (P25-P50)**

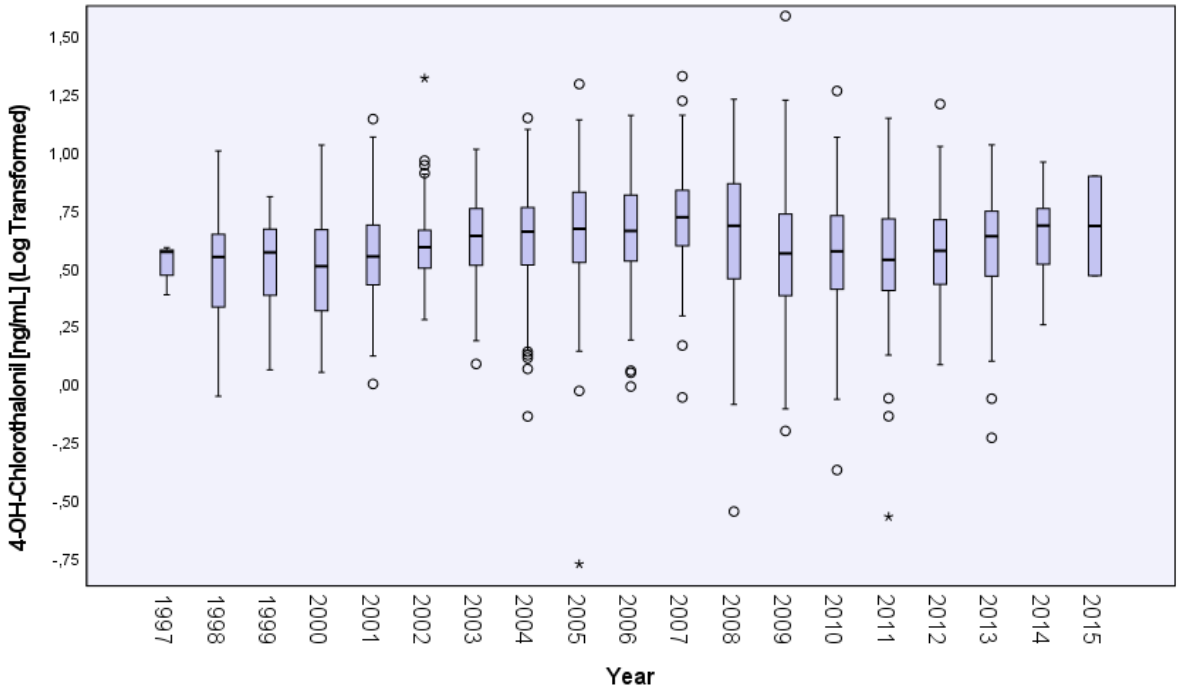

Figure 2. Timeline series (1997-2015) of blood serum concentrations of 4-OH-Chlorothalonil [ng/mL] (log-scale) in pregnant Swedish women in Scania plotted against sampling year (all values included).

Table 6. Analysis of temporal trend through linear regression

Annual change (β, % per year) in concentrations (log-transformed) of 4-OH-Clorothalonil in blood serum samples in pregnant Swedish women in Scania 1997-2015, for all samples. Not statistically significant ($p<0.05$). The coefficient of determination (R^2) was 0,000 for the regression model, indicating that the variance in concentration is explained by factors other than calendar year.

Biomarker	**β**	**95 CI (%)**	***p***	***R*²**
4-OH-CHT	0,000	-0,003, 0,003	0,908	0,000

A boxplot of 4-OH-CHT (non-transformed) visualizing the concentrations variations each month is shown in Fig. 3. and one showing seasonal changes is shown in Fig. 4. For the seasons significant differences in median concentrations were observed for all combinations except winter-summer (sign. differences seen between summer-spring, spring-winter, winter-autumn, autumn-spring, and autumn-summer), Fig. 4.

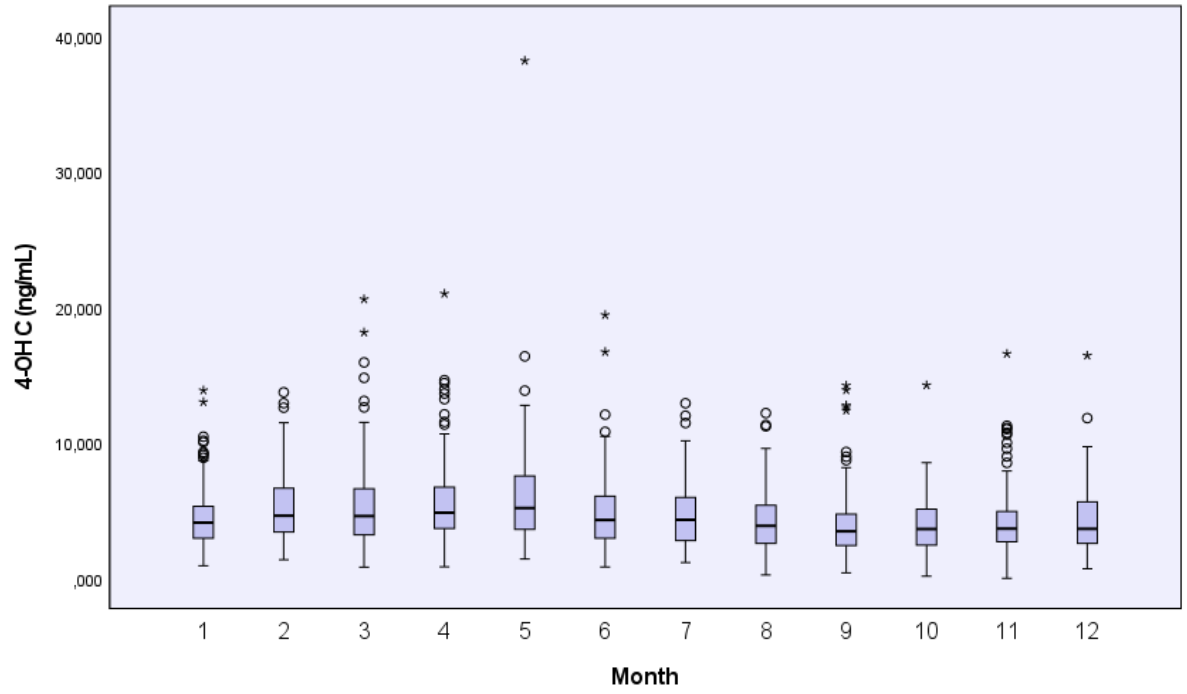

Figure 3. Timeline series (1997-2015) of blood serum concentrations of 4-OH-Chlorothalonil [ng/mL] (non-transformed) in pregnant Swedish women in Scania plotted against month (all values included). 1 represents January, 2 February and so on.

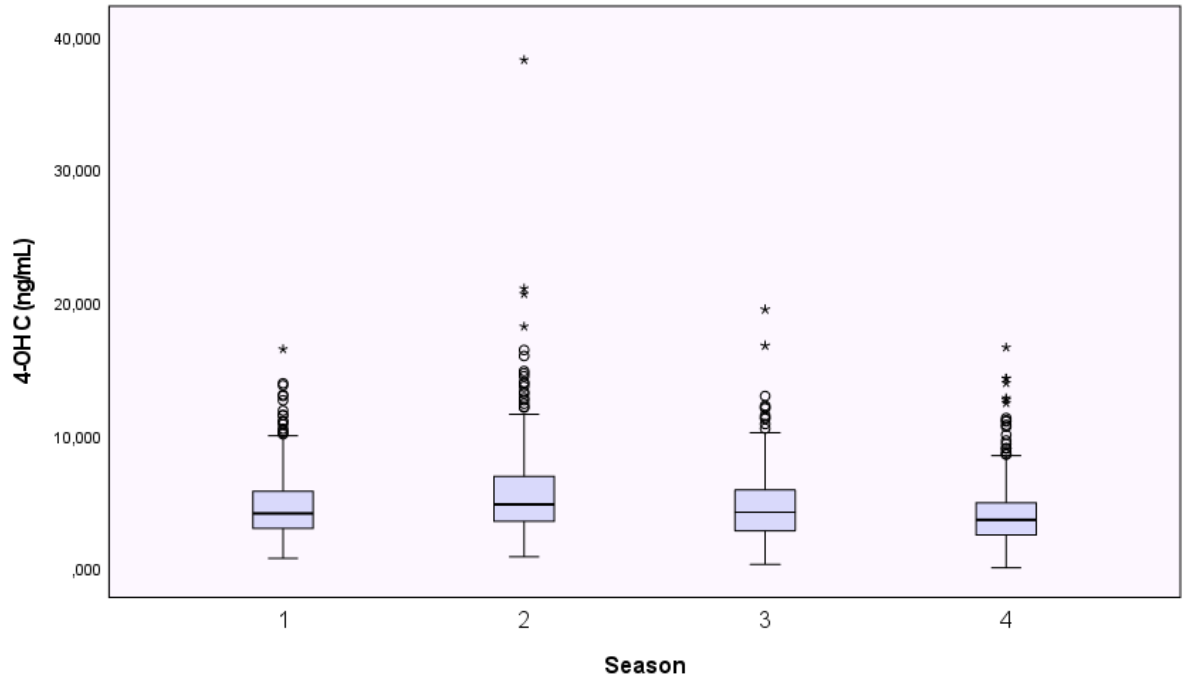

Figure 4. Timeline series (1997-2015) of blood serum concentrations of 4-OH-Chlorothalonil [ng/mL] (non-transformed) in pregnant Swedish women in Scania plotted against season (all values included). 1 represents the winter-distribution, 2 spring, 3 summer and 4 autumn.

Discussion

Time trend and concentrations of 4-OH-CHT in serum samples

Detection of 4-OH-CHT in serum of pregnant women from the years 1997 to 2015 was not expected, as use of chlorothalonil has not been approved for plant protection in Sweden since 1990. Even though CHT has been found in a few imported EU-grown vegetables during the period of the sampling it is most unlikely to be the cause of these results. Unfortunately the concentrations are not comparable due to different measurement methods and thus units but CHT found in one or two products one or two times (There is not data for all of these eighteen years) must be considered to be highly unlikely to impact 100% of the samples over 18 years of time (Livsmedelsverket, 2014).

An interpretation of the seasonal and monthly analysis can be made first after more research regarding possible factors impacting exposure during different months and seasons has been carried out. This is interesting because there are significant differences between for ex. spring and autumn concentrations throughout the serum samples, but not summer-winter. The high detection frequency (100% >LOD) of 4-OH-CHT in this population of pregnant women from Sweden 1997–2015 indicates exposure to the fungicide CHT or its breakdown product 4-OH-CHT.

While the use of CHT for agricultural purposes is banned, it is still allowed (in small concentrations) as paint additive and wood preservative (Pesticide Properties DataBase (PPDB), 2022; The Californian Office of Environmental Health Hazard Assessment (OEHHA), 2022). Additionally, it was still allowed in many countries inside and outside the EU during the serum sampling of this study (where it was first banned 2019) (Regulation 2019/677). CHT in serum might therefore be connected to the consumption of imported food or products from abroad containing CHT. Further, the National Food Agency in Sweden has reported that chlorothalonil occasionally exceeded the maximum permitted level in some imported fruit (Livsmedelsverket, 2014).

In the environment, 4-OH-CHT appears to be more persistent, mobile, and toxic than CHT [Chaves 2008, Cox 1997]. It has been reported to be slightly toxic to aquatic organisms and moderately toxic to birds and mammals [Raman 2014]. Possibly, humans are expose not to CHT, but to its metabolite 4-OH-CHT. This might occur if CHT brakes down in foodstuff or products containing CHT before human exposure occurs. More research is needed to further explore possible sources of exposure to CHT and 4-OH-CHT.

Strengths and limitations of the study

This study is unique in covering human serum concentrations of the fungicide CHT over an extensive period of 18 years in pregnant women. It is a large study that includes biological samples from many individuals from the population. The long-sample storage up to 25 (samples analysed in 2022) years could impact the quality of the samples and the stability of the biomarker. This data may imply that the biomarker 4-OH-CHT may be stable at −20 °C for at least 25 years allowing its use as a specific biomarker for CHT exposure, but this needs to be examined by measuring 4-OH-CHT at sampling or any reference point to be able to compare the concentrations. A possible degradation in concentration will thus show at a later measurement.

Only pregnant women were recruited during the 18 sampling years. It is plausible that the exposure levels of other females and/or men might differ from those of pregnant women. It is possible that women in general eat more fruits and vegetables compared to men (Wallstorm, 2000). Pregnant women might even eat healthier compared to non-pregnant women. Therefore, we cannot claim that our population presents the general population. However, to the best of our knowledge, this is the first report on 4-OH-CHT in human serum. While non-targeted screening approaches have revealed the presence of 4-OH-CHT in human blood [Plassmann, 2018] and in breast milk [Baduel, 2015; Pourchet, 2021], this is the first biomonitoring study of a CHT metabolite in humans.

Conclusion

Unexpectedly, we detected the biomarker 4-OH-CHT in measurable concentrations in 100% of blood serum samples, even though CHT has not been permitted for agricultural use in Sweden since the 1990s. This indicates a widespread and continuous exposure of at least parts of the Swedish population. Furthermore, we found no time trend for 4-OH-CHT over time according to year. There is a seasonal difference in concentration median for all seasons except summer-winter. Swedish pregnant women have a continuous exposure of CHT or its metabolite 4-OH-CHT which seems stable over time (1997-2015). This population has a median concentration of 4,1 ng 4-OH-CHT/mL serum. Comparable measurements of the metabolite 4-OH-CHT in human samples have, to our knowledge, not been published elsewhere.

References

Armbrust, K. L. (2001). Chlorothalonil and chlorpyrifos degradation products in golf course leachate. *Pest Management Science: formerly Pesticide Science*, *57*(9), 797-802. https://doi.org/10.1002/ps.361

Battaglin, W. A., Kuivila, K., Winton, K., & Meyer, M. (2008). *Occurrence of chlorothalonil, its transformation products, and selected other pesticides in Texas and Oklahoma streams, 2003-2004*. U. S. Geological Survey Scientific Investigations Report 2008–5016, 9 p.

Chaves, A., Shea, D., & Cope, W. G. (2007). Environmental fate of chlorothalonil in a Costa Rican banana plantation. *Chemosphere*, *69*(7), 1166-1174. https://doi.org/10.1016/j.chemosphere.2007.03.048

Croucher, L., & Jewess, P. (2007). *Metabolic pathways of agrochemicals: part 2: insecticides and fungicides*. Royal Society of Chemistry.

Farag, A. T., Karkour, T. A. Z., & Okazy, A. E. (2006). Embryotoxicity of oral administered chlorothalonil in mice. *Birth Defects Research Part B: Developmental and Reproductive Toxicology*, *77*(2), 104-109. https://doi.org/10.1002/bdrb.20074

Gamble, D. S., Lindsay, E., Bruccoleri, A. G., Langford, C. H., & Leyes, G. A. (2001). Chlorothalonil and its 4-hydroxy derivative in simple quartz sand soils: a comparison of sorption processes. *Environmental science & technology*, *35*(11), 2375-2380. https://doi.org/10.1021/es001864n

Galarnyk, M. (2018, 12 september), *Understanding Boxplots.* Towards Data Science. https://towardsdatascience.com/understanding-boxplots-5e2df7bcbd51

Ganzleben, C., Antignac, J.P., Barouki, R., Castaño, A., Fiddicke, U., Klánová, J., Lebret, E., Olea, N., Sarigiannis, D., Schoeters, G. R., Sepai, O., Tolonen, H. & Kolossa-Gehring, M. (2017). Human biomonitoring as a tool to support chemicals regulation in the European Union. *International Journal of Hygiene and Environmental Health*, *220*(2), 94–97. https://10.1016/j.ijheh.2017.01.007

Hendryx, M. & Luo, J. (2018). Children's environmental chemical exposures in the USA, NHANES 2003–2012. *Environmental science and pollution research*, *25*(6), 5336–5343. https://10.1007/s11356-017-0874-5

Hou, F., Zhao, L., & Liu, F. (2016). Residues and dissipation of chlorothalonil and azoxystrobin in cabbage under field conditions. *International journal of environmental analytical chemistry*, *96*(12), 1105-1116. https://doi.org/10.1080/03067319.2016.1233969

International Programme on Chemical Safety [IPCS]. (1996), Environmental health criteria 183: Chlorotlalonil, United Nation Environment Programme, International Labour Organisation World Health Organization. https://inchem.org/documents/ehc/ehc/ehc183.htm [Retrieved: 2022-02-23]

Jansson, A., Fohgelberg, P., & Widenfalk, A. (2015). *Kontroll av bekämpningsmedelsrester i livsmedel 2015*. [Control of pesticide residues in food 2015] (Report 19). Livsmedelsverket. [The national food administration.]
https://www.livsmedelsverket.se/globalassets/publikationsdatabas/rapporter/2016/kontroll-av-bekampningsmedelsrester-i-livsmedel-2015.pdf s.33

Jansson, A., Holmbäck, X., & Wannberg, A. (2009). *Kontroll av bekämpningsmedelsrester i livsmedel.* [Control of pesticide residues in food] (Report 17). Livsmedelsverket. [The national food administration.]
https://www.livsmedelsverket.se/globalassets/publikationsdatabas/rapporter/2011/2011_livsmedelsverket_17_bekampningsmedelsrester_2009.pdf

Jakubowski, M. & Trzcinka-Ochocka, M. (2005). Biological monitoring of exposure: trends and key developments. *Journal of Occupational Health, 47*(1), 22–48. https://10.1539/joh.47.22

Jordbruksverket. (2021*). Hur stor andel av livsmedlen som säljs på marknaden är producerade i Sverige?* Svensk marknadsandel 2020 (jordbruksverket.se)

Kemikalieinspektionen [KEMI]. (2021). Bekämpningsmedelsregistret, ämne, Klortalonil,

https://apps.kemi.se/BkmRegistret/Kemi.Spider.Web.External/Aemne/Details/674 [Retrieved: 2022-03-16]

Kwon, J.W. & Armburst, K.L. (2006). Degradation of chlorothalonil in irradiated water/sediment systems. *Journal of agricultural and food chemistry, 54,* 3651-3657. https://10.1021/jf052847q

Lewis, K.A., Tzilivakis, J., Warner, D. & Green, A. (2016). An international database for pesticide risk assessments and management. *Human and Ecological Risk Assessment: An International Journal*, *22*(4), 1050-1064. http://10.1080/10807039.2015.1133242

Lin, H., Zhao, S., Fan, X., Ma, Y., Wu, X., Su, Y., & Hu, J. (2019). Residue behavior and dietary risk assessment of chlorothalonil and its metabolite SDS-3701 in water spinach to propose maximum residue limit (MRL). *Regulatory Toxicology and Pharmacology, 107,* 104416. https://doi.org/10.1016/j.yrtph.2019.104416

Livsmedelsverket. (2014). *Kontroll av bekämpningsmedels rester i livsmedel 2011 och 2012* (Report 5). https://www.livsmedelsverket.se/globalassets/publikationsdatabas/rapporter/2014/2014-livsmedelsverket-5-kontroll-bekampnmed.pdf

Lopes, F. C., Junior, A. S. V., Corcini, C. D., Sánchez, J. A. A., Pires, D. M., Pereira, J. R., Gilberto Primel, E., Fillmann, G. & Martins, C. D. M. G. (2020). Impacts of the biocide chlorothalonil on biomarkers of oxidative stress, genotoxicity, and sperm quality in guppy Poecilia vivipara. *Ecotoxicology and environmental safety*, *188*, 109847. https://doi.org/10.1016/j.ecoenv.2019.109847

Lundqvist, A.-C., Andersson, S., & Lönn, M. (2008). *Genetic variation in wild plants and animals in Sweden: A review of case studies from the perspective of conservation genetics* (Report 5786). Swedish Environmental Protection Agency. http://www.naturvardsverket.se/Documents/publikationer/620-5786-2.pdf

Naturvårdsverket. (2007). *Tillsyn med framsyn: Naturvårdsverkets tillsynsvägledning 2007 – 2009* [Far-sighted monitoring: The Swedish Environmental Protection Agency's guidelines for how to conduct monitoring 2007 – 2009] (Report 5675). http://www.naturvardsverket.se/Documents/publikationer/620-5675-1.pdf

Plassmann, M.M., Fischer, S. & Benskin, J.P. (2018). Nontarget Time Trend Screening in Human Blood, *Environmental Science & Technology Letters, 5*(6), 335-340 http://10.1021/acs.estlett.8b00196

Pourchet, M., Narduzzi, L., Jean, A., Guiffard, I., Bichon, E., Cariou, R., Guitton, Y., Hutinet, S., Vlaanderen, J., Meijer, J., Le Bizec, B., & Antignac, J.P. (2021). Non-targeted screening methodology to characterise human internal chemical exposure: Application to halogenated compounds in human milk, *Talanta, 225*. https://10.1016/j.talanta.2020.121979.

Raman, P. (2014). Chlorothalonil. In *Encyclopedia of Toxicology* (3rd. Ed., pp. 919-922). Academic Press. http://10.1016/B978-0-12-386454-3.00283-9 https://www.sciencedirect.com/science/article/pii/B9780123864543002839 [Retrieved: 2022-03-16]

Ramírez-Bustos, I. I., Saldarriaga-Noreña, H., Fernández-Herrera, E., Juárez-López, P., Alia-Tejacal, I., Guillén-Sánchez, D., ... & López-Martínez, V. (2019). Dissipation behavior of three pesticides in prickly pear (Opuntia ficus-indica (L.) Mill.) pads in Morelos, Mexico. *International Journal of Environmental Research and Public Health*, *16*(16), 2922. https://doi.org/10.3390/ijerph16162922

Regulation 2019/677. *Concerning the non-renewal of the approval of the active substance chlorothalonil, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market, and amending Commission Implementing Regulation (EU) No 540/2011*. European Parliament, Council of the European Union. https://eur-lex.europa.eu/legal-content/ENG/TXT/HTML/?uri=CELEX:32019R0677

Regulation 2016/67. *Amending Annexes II, III and V to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residue levels for ametoctradin, chlorothalonil, diphenylamine, flonicamid, fluazinam, fluoxastrobin, halauxifen-methyl, propamocarb, prothioconazole, thiacloprid and trifloxystrobin in or on certain products*. European Parliament, Council of the European Union. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32016R0067&qid=1645623516195

Rouchaud, J. & Roucourt, P. (1988). Hydrolytic Biodegradation of Chlorothalonil in the Soil and in Cabbage Crops. *Toxicological & Environmental Chemistry*, *17*(1), 59-68. http://10.1080/02772248809357278

Sakai, M. (2002). Determination of pesticides and chronic test with Daphnia magna for rainwater samples. *Journal of environmental science and health, 37*(3), 247-254. http://10.1081/PFC-120003102

Shi, X. Z., Guo, R. J., Takagi, K., Miao, Z. Q., & Li, S. D. (2011). Chlorothalonil degradation by Ochrobactrum lupini strain TP-D1 and identification of its metabolites. *World Journal of Microbiology and Biotechnology*, *27*(8), 1755-1764. https://doi.org/10.1007/s11274-010-0631-0

Tillman, R.W., Siegel, M.K., & Long, J.W. (1973). Mechanism of Action and Fate of the Fungicide Chlorothalonil (2,4,5,6-Tetrachloroisophthalonitrile) in Biological Systems 1. Reactions with Cells and Subcellular Components of Saccharomyces pastorianus. *Pesticide Biochemistry and Physiology*, *3*(2), 160–167. https://10.1016/0048-3575(73)90100-4

The Californian Office of Environmental Health Hazard Assessment (OEHHA). (n.d.), Chlorothalonil. Retrieved May 13, 2022, from https://oehha.ca.gov/chemicals/chlorothalonil

US Environmental Protection Agency. (1999). Chlorothalonil: Reregistration Eligibility Decision (RED). EPA 738-R-99-004, Washington, DC.

Valverde-Garcia, A. G. P. E., Gonzalez-Pradas, E., Aguilera-Del Real, A., Ureña-Amate, M. D., & Camacho-Ferre, F. (1993). Determination and degradation study of chlorothalonil residues in cucumbers, peppers and cherry tomatoes. *Analytica chimica acta*, *276*(1), 15-23. https://doi.org/10.1016/0003-2670(93)85034-H

Wallstrom, P., Wirfalt, E., Janzon, L., Mattisson, I., Elmstahl, S., Johansson, U. & Berglund, G. (2000). Fruit and vegetable consumption in relation to risk factors for cancer: a report from the Malmo Diet and Cancer Study. *Public Health Nutrition, 3*(3), 263–271. http://10.1017/S1368980000000306

Xu, W., Vebrosky, E.N., & Armbrust, K.L. (2020). Potential toxic effects of 4-OH-chlorothalonil and photodegradation product on human skin health. *Journal of Hazardous Materials, 394.* https://10.1016/j.jhazmat.2020.122575

Yusa, V., Millet, M., Coscolla, C., & Roca, M. (2015). Analytical methods for human biomonitoring of pesticides. A review. *Analytical Chimica Acta*. *891*, 15–31. https://10.1016/j.aca.2015.05.032

Zhang, Q., Ji, C., Yan, L., Lu, M., Lu, C., & Zhao, M. (2016). The identification of the metabolites of chlorothalonil in zebrafish (Danio rerio) and their embryo toxicity and endocrine effects at environmentally relevant levels. *Environmental pollution*, *218*, 8-15. https://doi.org/10.1016/j.envpol.2016.08.026