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Exposure duration and absorbed dose assessment in pesticide-exposed agricultural workers: Implications for risk assessment and modeling

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ABSTRACT

Introduction: Absorbed dose assessment from dermal exposure involves multiplying skin contamination by the dermal absorption coefficient, which is usually defined for the standard workday of 8 h. This strategy may suffer from limitations when the duration of exposure is extremely variable, such as in agricultural exposure to pesticides.
Objectives: The aim of this study was to estimate the dose of mancozeb absorbed by agricultural pesticide applicators in a typical working day considering the real duration of exposure, to compare these estimates with those coming from the use of the Fixed Fractional Approach, and to assess the suitability of the dose estimates in the interpretation of biological monitoring results.
Methods: In a series of real-life field studies on 29 workers applying mancozeb in vineyards for 38 work days, three sets of data were collected: information regarding work activities for each work day, potential (on clothes) and actual skin exposure using the “patch” methodology, and excretion of ethylenethiourea (ETU) in the 24-h pre-exposure and 24-h post-exposure urine samples. The statistical analyses were done using the R Language and Environment for Statistical Computing.
Results: Accounting for the duration of exposure led to a substantial reduction in the absorbed dose estimates, compared to the estimates coming from the Fixed Fractional Approach. In particular, absorbed dose by the body, hands' and total absorbed dose were reduced by 50%, 81%, and 80% respectively. The body dose estimated considering both approaches still correlated better with post-exposure 24-h urine ETU levels than the hands' dose, although more than 90% of the estimated total absorbed dose comes from the hands.
Conclusions: An accurate estimate of the absorbed dose, carried out considering the real duration of exposure, can result in a higher correlation with a biomarker of occupational exposure, such as urine ETU, or at least yield more accurate results. This can facilitate the interpretation of biological monitoring data in pesticide-exposed agricultural workers despite the absence of biological exposure limits. ETU should be evaluated as a potentially relevant source of exposure due to ethylenebisdithiocarbamates’ (EBDCs) degradation in the formulated product or spray mixture.

1. Introduction

Dermal exposure represents a crucial route of exposure in various occupations, above all in the service sectors, manufacturing, and agriculture. Pesticides represent one of the main chemical groups, next to solvents and polycyclic aromatic hydrocarbons, where dermal exposure could result in adverse health effects. In open field farming, studies have shown that the inhaled amount is likely to be low compared to the amount deposited on the skin (Aprea et al., 2004; Vitali et al., 2009), and in even in greenhouses high dermal exposures can result from application or re-entry processes (Tielemans et al., 1999). Agricultural activities are also characterized by an instability of climatic and working conditions, use of complex pesticide mixtures, frequent disregard of good agricultural practices (GAP), and misuse, or even non-use, of personal protective devices (PPDs) (Arbuckle et al., 1999; Hines et al., 2001; Mandic-Rajcevic et al., 2015; Rubino et al., 2012).

Absorbed dose assessment done in the pre- and post-marketing phases involves estimating the skin dose and multiplying it by the

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dermal absorption coefficient defined for the standard work-day duration of 8 h (EFSA, 2014; Mandic-Rajcevic et al., 2013). This strategy, named the "*Fixed Fractional Approach",* is advocated by regulatory agencies, but may suffer from limitations in cases where the duration of exposure is extremely variable and the most common route of exposure is dermal contact (Frasch et al., 2013; Regulation, 2009). Previous field studies showing a contribution of above 90% of the hands' exposure to the total skin exposure have failed to find a good correlation between the hand exposure and measured urinary biomarkers (Fustinoni et al., 2014; Mandic-Rajcevic et al., 2018, 2015; Mercadante et al., 2018). It was hypothesized that the amount of active substance measured on the hands might only represent the exposure lasting until the performed hand-wash, and the workers wash their hands several times during the workday (Mandic-Rajcevic et al., 2018, 2015).

Estimating the dose absorbed by the workers and assessing their risk during application in different exposure scenarios is a challenging but necessary activity. Field studies are the gold standard in collecting realistic data on exposure and studies of associations between pesticides and various health outcomes have underlined the misclassification of exposure as one of the biggest challenges (Brouwer et al., 2016). Absorbed dose and risk assessment in typical use scenarios are critical for defining the main exposure determinants, controlling the occupational risk posed by pesticides, and improving models and tools for exposure and risk assessment in the pre- and post-marketing phases of active substances.

Ethylene-bis-dithiocarbamates (EBDC) are a group of fungicides widely used over the last few decades due to their low acute toxicity, short environmental persistence, and good fungicidal activity (Maroni et al., 2000). One of the most used compounds in this group, mancozeb, produces several metabolic products among which the most relevant is Ethylene-*bis*-thiourea (ETU), which is also a product of its environmental degradation (López-Fernández et al., 2016; Somerville, 1986). In plant protection products, only small amounts of ETU contamination have been found, and among EBDCs, the most susceptible to degradation is zineb, whilst mancozeb seems more stable (Camoni et al., 1988; Šovljanski and Živanović, 1984). ETU has already been measured in workers occupationally exposed to EBDCs (Colosio et al., 2002; Kurttio et al., 1990), and several studies have investigated and confirmed its validity as a biomarker of occupational exposure (Colosio et al., 2007, 2002).

The aims of this study were to estimate the dose of mancozeb absorbed by agricultural pesticide applicators in a typical working day considering the real duration of exposure, to compare the estimates obtained with those resulting from the use of the *Fixed Fractional Approach*, to assess the suitability of the produced dose estimates in the interpretation of biological monitoring results, and assess the risk in agricultural workers from ETU exposure due to the mancozeb's degradation in the product and its metabolization after absorption.

2. Material and methods

This study was carried out between April and July 2011 in Mantova and Pavia provinces of the Region of Lombardy (Northern Italy). Enterprises using mancozeb in vineyards were selected, methods and aims of the study were described to the employers and employees in dedicated meetings, and all participants signed the informed consent form. The Ethics Committee of the San Paolo Hospital (University of Milan teaching hospital) approved the study in February 2011.

2.1. Data and sample collection

Three sets of data were collected: (a) baseline characteristics of the study subjects and information regarding work activities for each work day using a targeted Data Collection Sheet developed for this activity (Mandic-Rajcevic et al., 2015); (b) potential (on clothes) and actual skin exposure by using the "patch" methodology and collecting the hand-wash liquid (OECD, 1997); (c) excretion of the mancozeb metabolite, ethylenethiourea (ETU) measured in the 24-h pre-exposure and 24-h post-exposure urine samples of the study group.

2.2. Sample analysis

The determination of free ETU in different kind of samples (pad, hand wash and urine) was obtained without hydrolysis using liquid chromatography-mass spectrometry, namely with Acquity UPLC system (Waters, Milford, MA, USA) coupled with a triple quadrupole Waters TQD mass spectrometer. ETU determinations in urine samples were done in line with previously published methods measuring only the free ETU (Fustinoni et al., 2005; Jones et al., 2010; Sottani et al., 2003), based on the good ETU signal due to the optimized ionization conditions in the positive ion mode (ESI+), as well as due to free ETU being the molecule of major toxicological concern. A recent paper underlinded a potential weakness in this approach, as it demonstrated that the free ETU in the urine might represent only 20% of the total ETU measured after hydrolysis (Ekman et al., 2013).

For quantitative analysis the TQD detector was used with an ESI interface in positive ion mode (ESI+). The MRM acquisition used to quantify free ETU was: m/z 103 → 44 (CV 36, CE 16); for internal standard $^2H_4$-ETU quantification was obtained in SIR: m/z 107 (CV35).

UPLC separation was performed on a Waters UPLC HSS T3 1.8 μm (2.1 × 100 mm) column kept at 28 °C, by gradient elution with a mixture containing variable proportion of water and methanol, delivered at a flow rate of 0.4 ml/min. The retention time of ETU and its internal standard was 1.3 min.

Urine samples (2 ml) were diluted with water (1 ml), spiked with $^2H_4$-ETU and purified using diatomaceous earth column (ChemElut® 3 ml unbuffered, Varian, Poole, UK) (Jones et al., 2010). In particular, after loading, analyte was eluted with dichloromethane (6 ml * 5), with an interval of ∼10 min between different aliquots; the eluate was evaporated to dryness under a gentle stream of nitrogen and reconstituted with 0.1% formic acid (2 ml) and finally injected onto the chromatographic system (3 μl). The calibration curve (constructed with a pool of urine of no-smoking subjects) was linear in the range 2.5–100 μg/l. Linearity was assessed by least squares linear regression using 3 calibration curves prepared on separate days from different urine samples. The variability of the method was evaluated by analysing, on 2 different days, 4 urine replicates spiked at 4 and 20 μg/L. The limit of detection (0.1 μg/l) was defined as the amount injected (from an extracted urine sample) that gave a signal equivalent to three times the baseline noise.

Clothes and skin pads samples (8 × 12.5 cm) were spiked with $^2H_4$-ETU, inserted in a polypropylene tube and desorbed with 8 ml of water, vortexed for 10 min, centrifuged and an aliquot was injected onto UPLC after a suitable dilution factor with 0.1% formic acid solution. The calibration curve was linear in the range 1–50 μg for clothes pads and 5–500 ng for skin pads.

Hand wash samples were obtained with 25% isopropyl alcohol (500 mL*2); an aliquot was centrifuged, diluted 1:20 in 0.1% formic acid (1 ml), spiked with $^2H_4$-ETU and finally injected in UPLC (3 μl). The calibration curve for these samples was linear in the range 0.2–4 mg.

For all the type of samples two quality control were run after every ten samples; the recovery was at least > 77% for urine samples and > 86% for the other type of samples; percent CV was always lower 15%. The laboratory procedure for each kind of sample is presented in the Supplementary Table 1.

2.3. Absorbed dose and risk assessment using the fixed fractional absorption approach

The absorbed dose of the active substance per kilogram of workers' body weight (per kg bw) was first calculated using the fixed fractional absorption approach:

$$Absorbed_{kg\ bw} = \frac{Exposure\ \times Dermal\ Absorption}{Body\ Weight}$$

Where ***Absorbed**$_{kg\ bw}$* was the absorbed dose of a mancozeb per kilogram of body weight, ***Exposure*** was the assessed skin exposure from patches for the body and collected hand-wash licquid for the hands, ***Dermal Absorption*** was the coefficient of dermal absorption established for mancozeb in the authorisation process (SANCO, 2009), and ***Body Weight*** was the actual body weight of each participating worker.

Finally, the risk was calculated as the saturation of the Acceptable Operator Exposure Level (AOEL), expressed in percentages, as:

$$Risk = \frac{Absorbed_{kg\ bw}}{AOEL_{mancozeb}} \times 100\%$$

The absorbed dose was calculated separately for the body and hands', and then summed to yield the total absorbed dose.

2.4. Absorbed dose adjusted for the duration of exposure

The duration of the workday was considered equal to the duration of body exposure in our calculations. The median duration of body exposure (workday) was 4 h, with individual values ranging from 2 h up to 13 h. The agricultural workers participating in our study washed their hands from 1 to 6 times during the workday, with a median of 2 times. The duration of hand exposure in our calculations was the duration of work before a handwash for each worker/work day. Its median value was just above 1 h and a half, with individual values ranging from around 50 min to up to 6 and a half hours.

To take into account the duration of body and hands' exposure for each worker, we applied the first order reaction formula to each calculated body exposure and each hand exposure sample:

$$Absorbed_t = Exposure_0 - Exposure_t$$

$$Exposure_t = Exposure_0 \times\ e^{-k\Delta t}$$

thus:

$$Exposure_0 = \frac{Exposure_t}{e^{-k\Delta t}}$$

and:

$$Absorbed_t = \frac{Exposure_t}{e^{-k\Delta t}} - Exposure_t$$

where:

Absorbed**$_t$* was the calculated absorbed dose for each worker, taking into account the duration of exposure; ***Exposure**$_t$* is the exposure estimated by the "pad" and hand-wash methods for the body and hands, respectively. ***k is the kinetic absorption constant, characteristic for each active substance and calculated using its dermal absorption coefficient (0.24% for mancozeb), considering that the duration of exposure used to define it in the authorisation process was 8 h ($\Delta t = 8$) (SANCO, 2009). ***Exposure**$_0$* is the exposure at the beginning of absorption, that to which we consider the worker was exposed for the whole duration before a handwash, to avoid underestimating the absorbed dose.

k value was calculated using the following formula:

$$e^{-k\Delta t} = 0.0024$$

$$k = 0.75$$

The adjusted absorbed doses were calculated for each worker, taking into account his workday duration for the body dose, and the estimated amount of mancozeb in each hand wash sample and the duration of exposure to this amount for the hands' dose. The body and hands' doses were summed and then divided by the workers' body weight to yield the total adjusted dose (per kilogram of body weight).

2.5. Risk assessment for ETU exposure due to mancozeb degradation and metabolism

ETU can be found in EBDC products such as mancozeb due to spontaneous degradation. Values ranging from 0.03 to 1.48% have been reported in the literature (Camoni et al., 1988; Šovljanski and Živanović, 1984). Some authors report a slow percutaneous absorption of ETU from intact skin, although uptake from abraded skin appears to be rapid, and a more recent study found that around 10% of the applied dermal dose was excreted in urine (Ekman et al., 2013; Teshima et al., 1981). A dermal absorption study in rats proposed the dermal absorption factor of 26% (US Environmental Protection Agency, 2005). The acceptable daily intake (ADI) estimated for humans is 0.004 mg/kg bw (JMPR, 1993). There is no AOEL for ETU, as it is not a plant protection product. The Health Council of the Netherlands recommended a health-based occupational exposure limit based on the Council's Dutch Expert Committee on Occupational Standards (DECOS). They recommend the occupational exposure limit of 0.024 mg/m^3 as an 8-h time weighted average concentration. This would result in a dose, considering 100% absorption through the respiratory tract, an 8-h moderate work activity, and a 70 kg man (conservative estimates), of 0.005 mg/kg bw. In our calculations and low-tier risk assessment we use the ADI of 0.004 mg/kg bw as a more conservative limit.

Risk assessment for ETU exposure due to degradation in the product was performed using the following formula:

$$Risk = \frac{\text{Exposure}_{Mancozeb} \times \text{Degradation}_{Mancozeb \rightarrow ETU} \times \text{Dermal Absorption}_{ETU}}{\text{Body Weight} \times \text{ADI}_{ETU}}$$

where:

Risk is expressed as the saturation of ADI (%), ***Exposure**$_{Mancozeb}$* is the estimated exposure to Mancozeb, ***Degradation**$_{Mancozeb}$* is the degradation of 1% conservatively selected based on the above-cited literature, ***Dermal Absorption**$_{ETU}$* is the dermal absorption coefficient for ETU of 26%, selected conservatively based on the above-cited rat study, ***Body Weight*** is the weight of the worker in kilograms, and ***ADI**$_{ETU}$* is the Acceptable Daily Intake for ETU of 0.004 mg/kg bw.

Risk assessment for ETU measured in urine, due to the metabolism of the absorbed mancozeb and environmentally-formed ETU was performed using the following formula:

$$Risk = \frac{\text{ETU}_{Urine}}{\text{Body Weight} \times \text{ADI}_{ETU}}$$

Where:

Risk is expressed as the saturation of ADI (%), ***ETU**$_{Urine}$* is the measured amount of ETU in the 24-h urine sample of the workers, ***Body Weight*** is the weight of the worker in kilograms, and ***ADI**$_{ETU}$* is the Acceptable Daily Intake for ETU of 0.004 mg/kg bw.

2.6. Data processing and statistical analysis

Data processing and statistical analyses were done in the ***R language and Environment for Statistical Computing*** (R Core Team, 2017). Categorical data was presented by the number of observations followed by the percentage of the total. Continuous data was first plotted, and the distribution of variables of interest was tested using the Shapiro-Wilk test for normality. In case of deviation from the normal distribution, median, minimum, and maximum values were reported in text, non-parametric statistical tests, such as Mann-Whitney and Kruskal-Wallis tests, were used to compare differences between groups, and Spearman's rank-order correlation was used to determine if there was an association between the estimated absorbed doses and the levels of ETU excreted in 24-h urine, and the Spearman's rank correlation coefficient (Spearman's rho, ρ_s) is reported in text and figures.

3. Results

Twenty-nine healthy, right-handed male farmers, participated in this study, using mancozeb in vineyards for a total of 38 workdays. The workers used closed and filtered tractors (CFT) in 29 workdays and open tractors (OT) in 9 workdays. The median covered area during one workday was 6 ha, ranging from 1 to 20 ha. As far as personal protective devices (PPDs) are concerned, most workers used new mono-use coveralls and regular clothes (not work or protective clothing) below the coverall. Gloves were available in all cases when OTs, and in most cases when CFTs were used. The material of the gloves was in most cases rubber, followed by neoprene and latex. The detailed description of worker and workday characteristics, as well as the PPDs' availability and use, can be found in a previously published paper dealing with exposure determinants and the influence of PPDs on exposure levels (Mandic-Rajcevic et al., 2018).

3.1. Absorbed dose estimate through the fixed fractional approach, risk assessment, and biological monitoring

The median total dose of mancozeb was lower than 3 ng/kg bw when the fixed fractional approach was used, with a somewhat higher median value of 8 ng/kg bw for OTs and lower median value of 2 ng/kg bw for CFTs. Fig. 1 shows the absorbed dose of mancozeb and the contribution of body and hands to the total dose. Fig. 1 ***(Panel B)*** demonstrates individual workdays, with hands and body contribution to the total absorbed dose (denoted with red and green color, respectively), and a horizontal line representing the median hands' contribution. The median contribution of hand exposure and dose to the total dose was close to 97%.

Compared to the AOEL of 0.02 mg/kg bw (20,000 ng/kg bw), the absorbed doses were largely below the limit set in the registration process. Median AOEL saturation was less than 0.01%, with a somewhat higher value for OTs of 0.02% and a lower value for CFTs of 0.006%. In general, our workers absorbed a dose that was more than 10,000 times lower than the limit proposed during the registration process for mancozeb. OT workers absorbed a dose that was more than 4000 times lower than this limit, while CFT workers' dose was more than 15,000 times lower. The highest exposed OT and CFT workers received doses between 200 and 280 times lower than the AOEL, respectively.

The median pre-exposure 24-h urine ETU levels were 0.93 and 0.51 μg/g of creatinine for OT and CFT workers respectively. The median post-exposure 24-h urine ETU levels were 3.02 and 2.06 μg/g of creatinine for OT and CFT workers respectively. The median difference in ETU levels between pre- and post-exposure 24-h urine was 1.27 μg, with a higher median value of 1.83 μg for OT, and lower median value of 1.22 μg for CFT workers. In several cases, the difference between pre- and post-exposure 24-h urine was negative (e.g., workers having lower ETU values after the occupational exposure).

3.2. Accounting for the duration of exposure

Fig. 2 shows the individual cases of workers' exposure, divided by the type of tractor, and the change in the absorbed body, hands' and total dose when the duration of exposure was accounted for. Due to a wide range of absorbed doses, the *y*-axis was log transformed. Both hands', as well as body absorbed dose, were lower, with the estimated hands' dose reduced by a median of 81% (ranging from 18% to 90%), and the estimated body dose reduced by a median of 50%. In most cases, the hands' and total dose estimates were reduced when accounting for the duration of exposure. The body dose estimate did decrease in most cases, but in several cases even increased, in workers working longer than 8 h during one workday. In these cases the body dose increased by 4–63% when adjusted for the duration of exposure.

Adjusting for the duration of exposure also resulted in some changes in the contribution of hand and body doses to the total absorbed dose. Fig. 3 shows the contribution of body and hands' dose to individual workdays with the contribution of hands' (***red***) and body (***green***) dose to the total dose. When the duration of exposure was taken into account for the hands dose estimate, the contribution of hands to the total absorbed dose was reduced to 89%, from original 97% when 8-h exposure was assumed (Fig. 3*, **Panel A***). In case duration of exposure was taken into account for both hands and body dose, the contribution of hands to

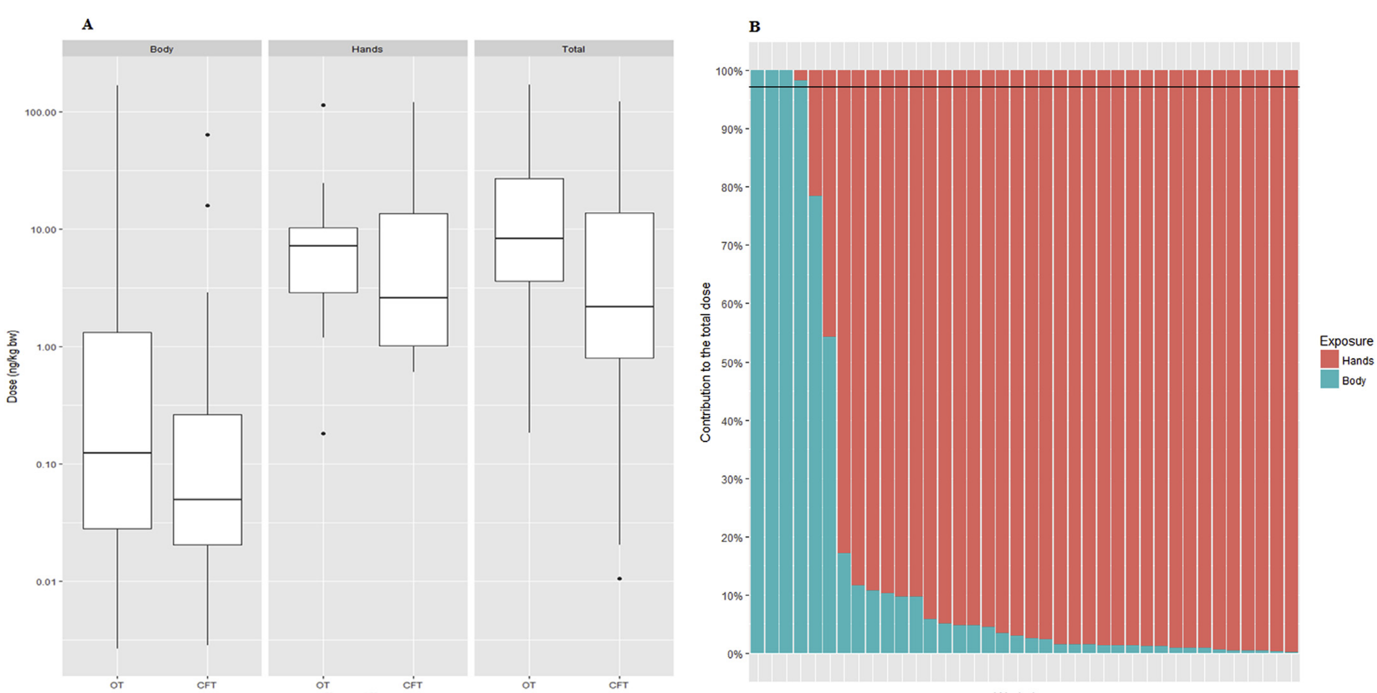

Fig. 1. Absorbed dose of mancozeb. ***Panel A***. Absorbed dose when a fixed (8-h) absorption coefficient is applied depending on the tractor type (OT – Open Tractor, CFT – Closed and Filtered Tractor). ***Panel B***. Contribution of body and hands exposure to the total absorbed dose when a fixed (8-h) absorption coefficient is applied.

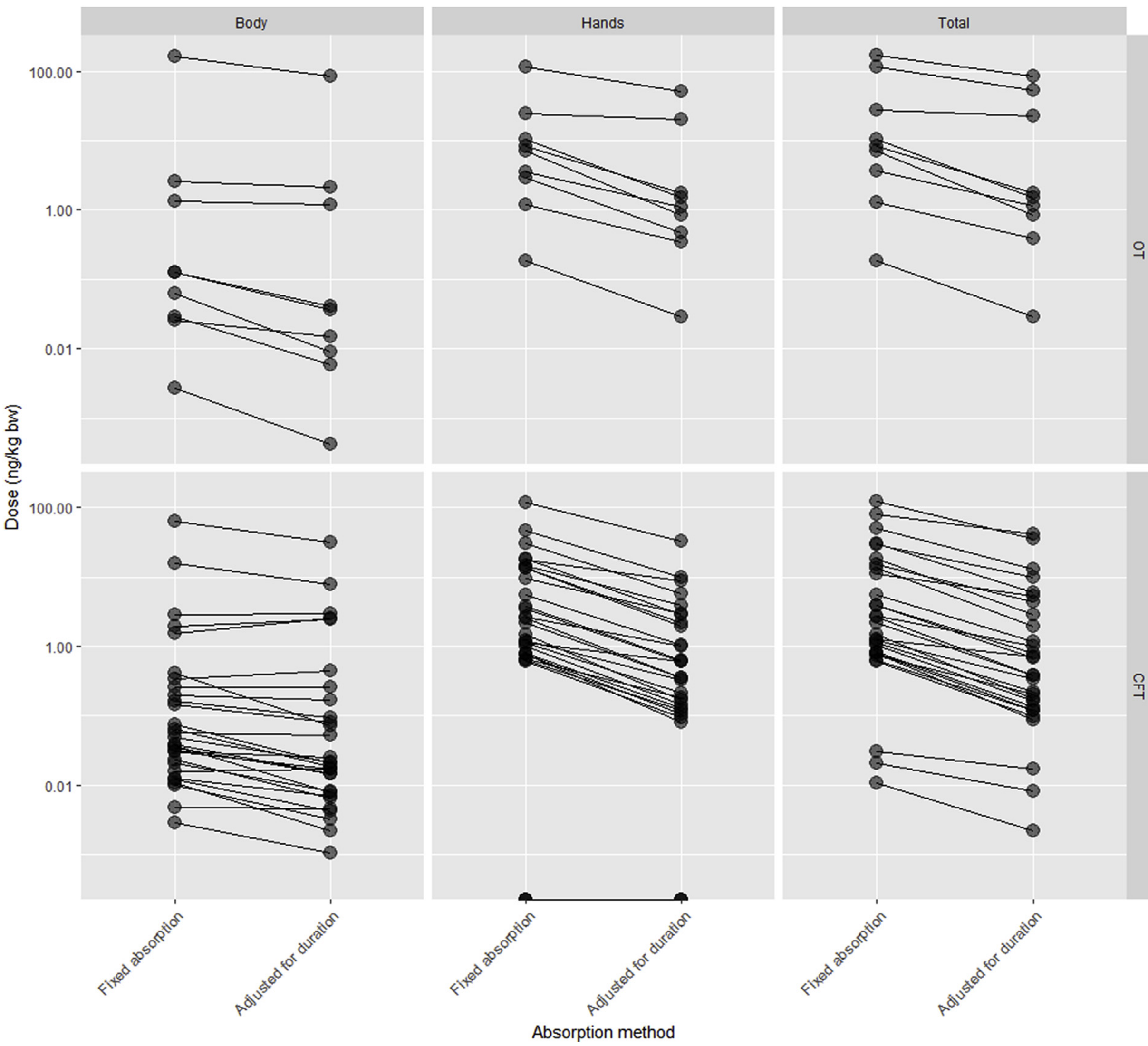

Fig. 2. Individual absorbed body, hands and total doses by type of tractor using a fixed (8-h) absorption coefficient and adjusted for the duration of exposure. OT – Open Tractor, CFT – Closed and Filtered Tractor.

the total absorbed dose was somewhat higher, around 92%, although still lower than the original 97% (Fig. 3, ***Panel B***). The risk assessment results for mancozeb exposure in OTs and CFTs did not change substantially by taking into account the duration of exposure considering the absorbed dose was already 10,000 times lower than the AOEL for mancozeb.

3.3. ETU as the biomarker of occupational exposure and correlation with the absorbed dose

The suitability of free ETU as a biomarker of mancozeb exposure was done by examining the correlations between ETU levels (post-exposure 24-h ETU with and without correction for creatinine and the difference between pre- and post-exposure 24-h ETU levels) and body, hands, and total absorbed dose, with and without adjusting for the duration of exposure. Fig. 4 shows the Spearman correlation coefficients between body, hands' and total doses (8-h and adjusted for the duration of exposure) and post-exposure 24-h ETU levels with and without correction for creatinine, or the difference between pre- and post-exposure 24-h ETU levels. For body dose, the highest correlation was found between the fixed duration estimate and the 24-h post-exposure ETU levels ($\rho_s = 0.58$). For hands' dose, the highest correlation was found between the hands dose adjusted for the real duration of exposure and the 24-h post-exposure ETU levels corrected for creatinine ($\rho_s = 0.41$). For the total dose, the highest correlation was found between the fixed duration estimate and the 24-h post-exposure ETU levels corrected for creatinine ($\rho_s = 0.51$). The correlation for hands' dose was improved when the real duration of exposure was taken into account.

3.4. Risk assessment for ETU exposure due to mancozeb degradation and metabolism

Risk due to ETU exposure was assessed considering the possible presence of ETU as a product of degradation of mancozeb, as well as the result of the metabolism of absorbed mancozeb. In both cases, worst-case scenario estimates (endpoints) were used, considering the available literature (see ***Material and Methods***). The median risk of ETU exposure due to its presence as a degradation product, expressed as the percentage of saturation of the Acceptable Daily Intake for ETU was below 0.1%. In Open tractors it ranged from 0.005% up to 4.5%, with a median value of 0.2%, while in closed and filtered tractor workers it ranged from 0.0003% up to 3.2%, with a median value of 0.06%. The median risk of ETU exposure based on free ETU levels measured in urine, due to the metabolism of mancozeb or absorbed environmentally-formed ETU was around 10 times higher, with a value of

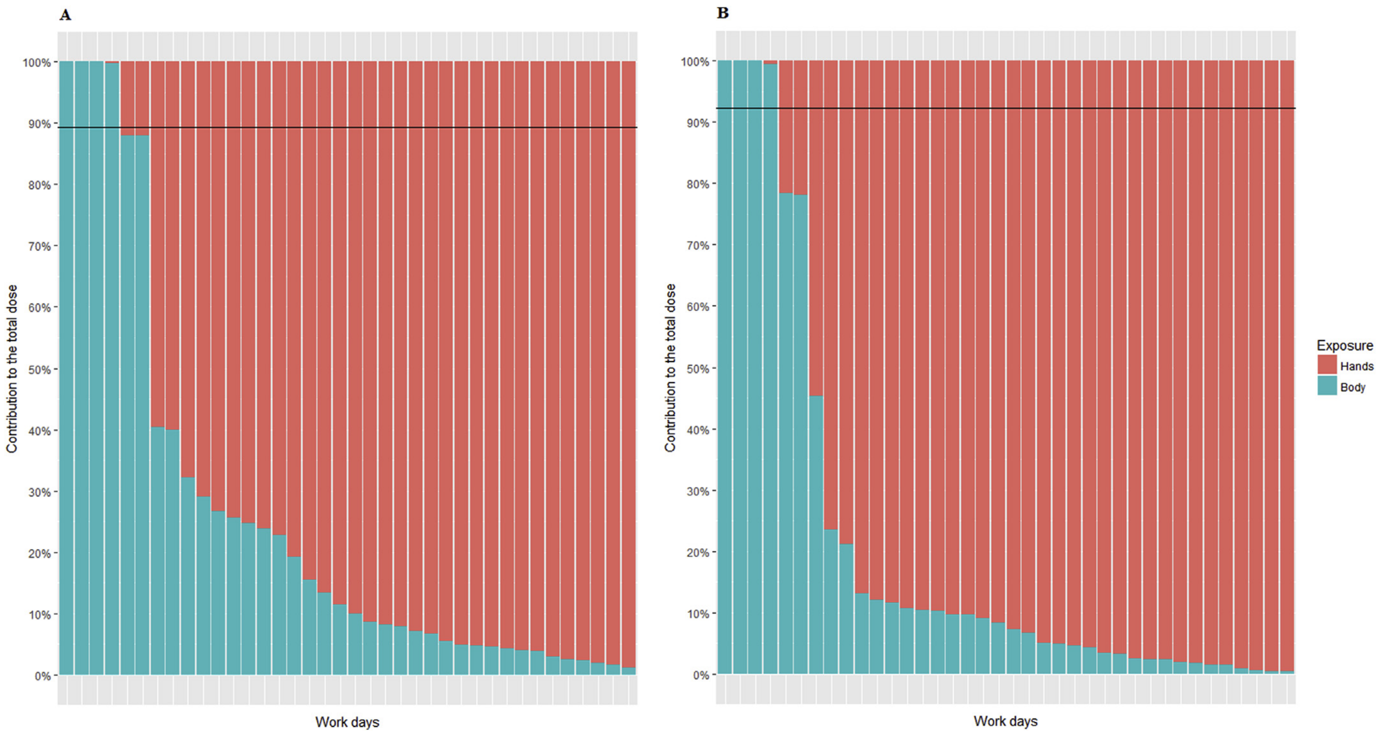

Fig. 3. Contribution of the body and hands' dose to the total absorbed dose when adjusted for the duration of exposure. ***Panel A*** - The absorbed hands' dose is estimated taking into account the duration of exposure. ***Panel B*** – Both the body and hands' absorbed dose is estimated taking into account the duration of exposure. The black line denotes the median hands' contribution to the total dose (89% and 92% in panels A and B, respectively).

0.81%. In open tractors, it ranged from 0.24% to 7.39%, with a median value of 0.78%, while in closed and filtered tractor workers it ranged from 0.10% to 5.23%, with a median value of 0.81%.

4. Discussion

This paper assesses pesticide applicators' mancozeb absorption in a typical working day, considering the real duration of exposure, and compares the results with those obtained through the application of the fixed fractional approach used in the regulatory exposure and risk assessment and in most, if not all, papers reporting results of pesticide field studies. Taking into account the real duration of exposure was achieved by applying the first order kinetic equation to mancozeb levels measured on body and hands and data on the duration of exposure collected in a field study conducted on a relatively large sample of pesticide applicators. To our knowledge, this paper represents the first practical attempt to improve pesticide exposure and risk assessment by systematically including the duration of exposure into the calculation of the absorbed dose using a novel statistical approach. This paper also evaluates the toxicological relevance of ETU forming through degradation of mancozeb, as well as by the metabolization of absorbed mancozeb, as a source of exposure.

Our study subjects were exposed to very low levels of mancozeb. Thus, their absorbed dose was, in most cases, several thousand times lower than the AOEL, and even the highest exposed workers received a dose several hundred times below the AOEL. Mancozeb is characterized by a relatively low dermal absorption coefficient of 0.24% and a relatively high Acceptable Operator Exposure Level of 0.035 mg/kg of body weight (SANCO, 2009). Our results indicate that, at least in the two studied exposure scenarios, the open tractor (OT) and closed and filtered tractors (CFT), there was no occupational health risk from mancozeb exposure for the workers. This favorable result is likely the consequence of strong regulation and enforcement of occupational safety and health standards found in Italy and Europe as a whole.

Connecting each measured exposure level to the individual duration of exposure resulted in a significant reduction in the absorbed dose estimates, when compared to the fixed fractional absorption approach. Namely, the dose absorbed by the body, hands and total absorbed dose were reduced by 50%, 81%, and 80% respectively (see Fig. 2). The most significant reductions were seen in absorbed hands' dose estimates, due to the high levels of exposure and the largest difference between fixed fraction assumption of an 8-h exposure, and the actual duration of exposure which was largely reduced by hand-washing done by the workers. Contrarily, the estimated absorbed body dose increased in several subjects, as their work continued beyond the 8-h used in the regulatory settings.

Collecting hand-wash to measure hands exposure is widely used in pesticide exposure and risk studies. This experimental procedure can mask the important practice of hand washing, and the approach of summing the measured values and applying the fixed absorption coefficient to the total hands' exposure, however straightforward, can and will lead to an overestimate of the absorbed dose. The contribution of 97% of the estimated hands' dose to the estimated total absorbed dose underlines the importance of correctly collecting, processing, and interpreting exposure monitoring results. Even when the duration of exposure is accounted for in the absorbed hands' dose estimates, hands remain the biggest contributor to the total dose. Nevertheless, our results suggest that even when the measured hand exposure is extremely high, it does not necessarily have led to a high absorbed dose, if the worker would wash his hands regularly during the workday (e.g., after the mixing and loading phase of work). This important hygienic practice represents, by our results, the simplest and most effective intervention to reduce the duration of hands' exposure to active substances, and in combination with the use of gloves could reduce the major part of the hand exposure and absorbed dose (Mandic-Rajcevic et al., 2018, 2015).

Among the 300 or so active substances registered in the European Union and the United States of America, many do not have such a low dermal absorption coefficient and a high AOEL as mancozeb. Studies have shown that, if the exposure scenario remains the same and a

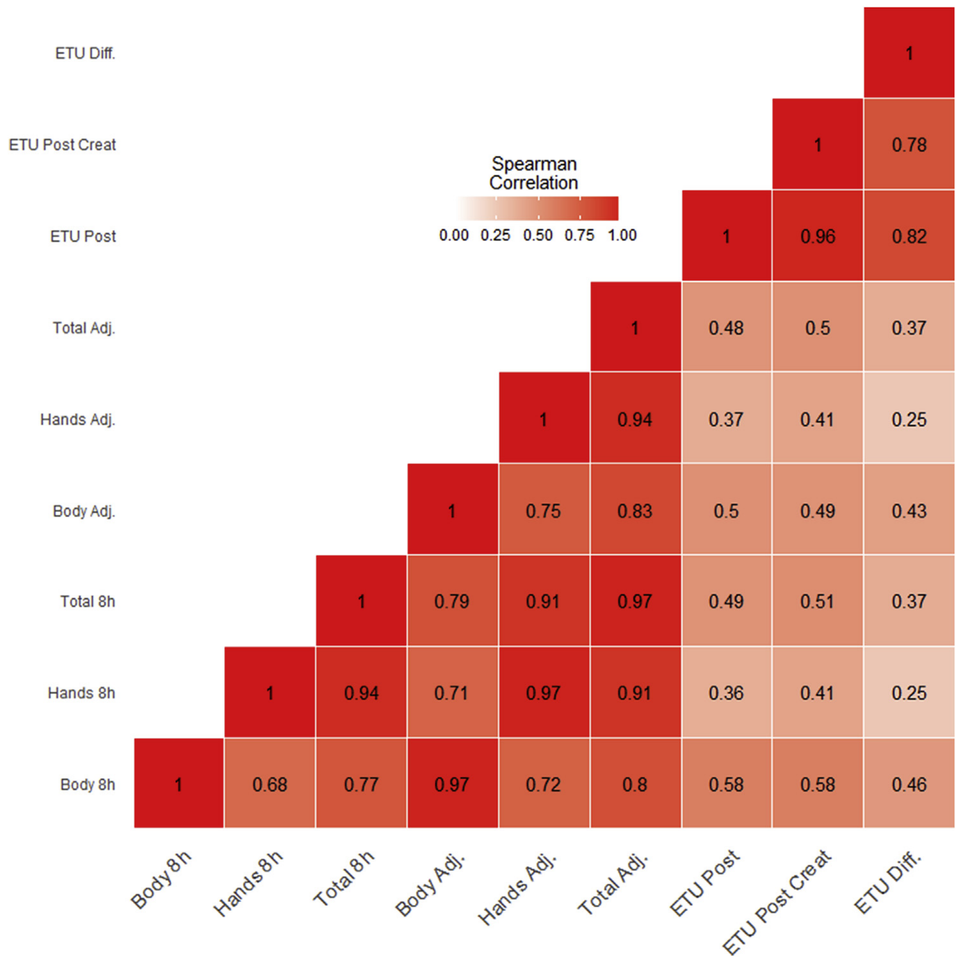

Fig. 4. Spearman correlation coefficients between estimated absorbed dose using the fixed (8-h) absorption coefficient and adjusted for the duration of exposure.

different active substance is hypothetically used in the amount recommended (e.g., by the Good Agricultural Practices), the risk estimates can change greatly, due to the changes in the dermal absorption and AOEL of the hypothesized active substance (Lundehn et al., 1992; Mandic-Rajcevic et al., 2015; Van Hemmen, 2001). In these cases, the reduction of the total absorbed dose by 50% can make a difference between a relevant and an irrelevant absorbed dose, with important implications for epidemiological studies and modeling.

The absorbed body dose in our study correlated better with post-exposure 24-h free ETU urine levels than the hands' dose, which is unexpected when considering that most of the estimated total absorbed dose comes from the hands, but is in line with previously published studies (Fustinoni et al., 2014; Mandic-Rajcevic et al., 2018, 2015). Adjusting the estimated absorbed doses for the duration of exposure did not improve the correlations between the absorbed doses (body, hands, and total) and post-exposure urine free ETU levels, but neither did it worsen it.

ETU has been suggested as an indicator of occupational and environmental exposure to EBDCs, although the problem of a lack of reference values has been underlined in previous studies (Aprea et al., 1997; Colosio et al., 2006). In our study, around 1 µg of free ETU was measured in pre-exposure urine, thus suggesting the presence of an underlying environmental exposure to EDBCs, most likely contributed by residues in food and wine, as highlighted by previous studies and belongs to the range of previously identified levels among the North Italian general population (Aprea et al., 1997; Colosio et al., 2006). The post-exposure urine ETU levels of 2–3 µg reflect the relatively low estimated absorbed doses in our workers. The median difference between pre- and post-exposure urine ETU levels of just below 2 µg shows that the occupational exposure did occur, but the increase in ETU was still below the 90th percentile for urine ETU found in the general population (Colosio et al., 2006). Observing a "negative difference" between the pre- and post-exposure urine ETU levels suggests that the workers were so well protected that their occupational exposure was much lower than the environmental exposure.

Free ETU in urine has commonly been used as a biomarker of occupational exposure to EBDCs (Colosio et al., 2002; Corsini et al., 2005; Liu et al., 2003), but has rarely been considered as a source of health risks except in industrial settings (Aprea et al., 1998). The United States Occupational Safety and Health Administration (US OSHA) classified ETU as a suspect carcinogen and National Institute for Occupational Safety and Health (NIOSH) recommends lowest feasible exposure, without setting any occupational exposure limit. The International Agency for Research on Cancer has classified ETU as Group 3 – "Non classifiable as to its carcinogenicity to humans" (IARC Monograph Vol. 79 (2001) p. 659). Compared to mancozeb, ETU has a much higher dermal absorption coefficient, and a much lower limit dose. It's degradation in a formulated product is expected to be relatively low, with literature data suggesting it would be much lower than 1% (Camoni et al., 1988; Lo and Ho, 1993; Šovljanski and Živanović, 1984). The low-tier (worst-case scenario) risk assessment we performed for ETU exposure due to mancozeb degradation in the formulated product and metabolized absorbed mancozeb has demonstrated that, in our workers, the dose of ETU was from several thousand to several hundred times lower than the ADI. The exposure due to the metabolism of absorbed mancozeb and environmentally-formed ETU appears much more relevant, with median doses around 10 times higher than those due to degradation. The highest dose of ETU to which our workers have been exposed was almost 15 times lower than the ADI for ETU. Regardless of numerous papers describing EBDC exposure in agricultural settings, no paper, to our knowledge, has evaluated direct exposure to ETU due to EBDC degradation in the spray liquid or the metabolism of absorbed EBDCs. It is important to note that our method measured only free ETU in urine samples and may have underestimated the ETU levels. A recent paper found that free ETU represented only 20% of total ETU measured in urine samples of one male and one female subject, and the relationship between the hydrolysed and non-hydrolysed urine samples showed a linear trend and a relatively high variability between the samples and the subjects (Ekman et al., 2013). This indicates that total ETU levels of our study participants might be several times higher, bringing the exposure to ETU closer, but still below, the ADI for ETU. In order to avoid underestimating the absorbed dose, hydrolysis and measurement of total ETU is advised. Future studies should focus on the possibility of relevant ETU exposure and health risks in agricultural EBDC sprayers, considering that products other than mancozeb might produce higher ETU levels due to degradation (Šovljanski and Živanović, 1984), and that the rate of degradation of EBDCs in the diluted product might reach even higher levels, depending on the time (López-Fernández et al., 2016). The discussion on free and conjugate ETU found in urine samples, and the lack of information regarding the toxicological properties of conjugate ETU underline the importance of future research in this area. Finally, ETU represents the most relevant candidate for the development of a biological exposure limit for mancozeb, and potentially a common biological exposure limit for EBDCs.

Taking into account the duration of exposure is just one of the improvements which could be implemented in the estimation of the absorbed dose of active substances when non-standard (8-h) workdays characterize the work. The estimation of the absorbed dose through hand exposure can also be greatly influenced by the "loading effect," with the "load" defined as the mass of chemical applied per unit area of exposed skin (Kissel, 2011). It is unlikely that all of the active substance measured in the hand-wash liquid was in contact with the skin of the hands during the whole time between washing and available for absorption. Unfortunately, it is also likely that agricultural workers do not remove all their pesticide-contaminated clothes at the exact moment the work stops, nor do they immediately shower and wash their hands thoroughly to remove all residues of active substances on their skin. Therefore, in many cases, pesticide absorption may continue after the removal of the load. By accounting for the duration of exposure, we have attempted to apply one possible solution to one of the problems mentioned above, while the other factors and several models which could represent solutions have been discussed in detail elsewhere, and should be addressed in future studies (Frasch et al., 2013; Semple, 2004).

5. Conclusions

Accounting for the duration of exposure in pesticide application and other real-life conditions characterized by a non-standard (8-h) workday or hand washing can significantly reduce the overestimation of absorbed dose. In higher tier risk assessment, improvements in exposure and absorbed dose assessment can make a difference between evaluating an occupational situation as unsafe or not, especially when applying the exposure and risk scenario to a more toxic active substance. Furthermore, correctly correlating work situations with "real" risk is necessary for epidemiological studies of occupational health outcomes, where the problem of misclassification of exposure-related risk is a prominent source of uncertainty, especially in agricultural settings, has been underlined as the biggest challenge. Finally, re-analysis of existing data collected in numerous pesticide studies with improved methods for exposure and absorbed dose assessment could facilitate robust risk assessment of pesticide use and demonstration of the efficacy of protection strategies, with the final aim to render the unavoidable pesticide application risk-free for agricultural workers around the world.

Declarations of interest

None.

Conflicts of interest

The authors declare NO competing financial interest in relation to the work described.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2019.01.006.

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