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Longitudinal Assessment of Chlorpyrifos Exposure in Farmers and Residents of an Italian Alpine Region

Giuseppe Paglia[1] · **Fabiola M. Del Greco**[2] · **Cristoph Carli**[3] · **Baldur B. Sigurdsson**[4] · **Sigurdur Smarason**[2] · **Mario Wegher**[5] · **Cristian Pattaro**[2] · **Lino Wegher**[3]

Abstract

The aim of this study was to obtain a longitudinal evaluation of the exposure to chlorpyrifos (CP) and chlorpyrifos-methyl (CPM) in agricultural workers in South Tyrol and in a residential group living in the same area. CP and CPM are widely used pesticides in agriculture. Biological monitoring of CP and CPM exposure in humans can be achieved by analyzing urinary levels of 3,5,6-trichloro-2-pyridinol (TCPy). TCPy a metabolite of CP and CPM which is produced by a two-step metabolic transformation. Between May 14th, 2014 and March 16th, 2015 we conducted a longitudinal study on 28 farmers actively working in spray pesticide treatment and 43 non-farmers living in the same agricultural area of South Tyrol (Italy). Urine samples were collected at two time points: during the pesticide treatment period and in a temporally distant season that should guarantee metabolite clearance. We developed and validated a liquid chromatography-tandem mass spectrometry (LC–MS/MS) method for the determination of urinary TCPy levels. During the treatment season, both farmers and residents showed higher TCPy levels (median = 6.8 and 6.73 ug/g creatinine, respectively) than during the non-treatment season (median = 2.54 and 3.22 ug/g creatinine, respectively), suggesting a similar effect of the pesticide spraying on both groups. However, the observed TCPy levels resulted in a daily CP and CPM intake well below the limits recommended by FAO/WHO. During the non-treatment season, non-farmers showed higher TCPy levels values than farmers, suggesting the existence of TCPy of other unmeasured sources of exposure not considered in this study. This suggests that, for a comprehensive evaluation of the risks associated with TCPy exposure, additional sources should be identified in addition to CP and CPM pesticides.

Keywords Exposure · Chlorpyrifos · Chlorpyrifos-methyl · Biological Monitoring · LC–MS · South Tyrol

Introduction

Organophosphorus pesticides are widely used in agriculture, horticulture as well as for domestic and gardening worldwide. They were introduced to eliminate insects, but exposure of non-targeted species, including humans, may also occur. Therefore, they are matter of public concern, mainly due their negative impact on the wildlife and the environment and their adverse effect on health (Barr 2008).

Health outcomes associated with pesticide exposure is various, including the reproductive, digestive, endocrine, respiratory and neurological systems (Kamijima et al. 2004; Arcury et al. 2016; Jain 2017; Llop et al. 2017; Ye et al. 2017; Lerro et al. 2018).

Diet is probably the main source of exposure to pesticides in the general population (Becker et al. 2006; McKone et al. 2007), but exposure can also occur via direct skin contact and ihalation (Buck et al. 2001; Cattani et al. 2001).

Among the organophosphorus pesticides, chlorpyrifos (CP) and chlorpyrifos-methyl (CPM) are the most widely used insecticides worldwide (Eaton et al. 2008; Foxenberg et al. 2011; Foong et al. 2020). In particular, they were used in South Tyrol to prevent "apple proliferation". It is known

✉ Giuseppe Paglia
giuseppe.paglia@unimib.it

1 School of Medicine and Surgery, University of Milano-Bicocca, Milano, Italy

2 Institute for Biomedicine, Eurac Research, Bolzano, Italy

3 Südtiroler Sanitätsbetrieb, Azienda Sanitaria DellAlto Adige, Bolzano, Italy

4 Department of Clinical Biochemistry, Landspitali - University Hospital of Iceland, Reykjavik, Iceland

5 Center Agriculture Food Environment (C3A), University of Trento, Trento, Italy

that agricultural field workers can be exposed to significant amounts of CP (Fenske et al. 2013; Farahat et al. 2010; Crane et al. 2013; Singleton et al. 2015), however, exposure of general population individuals can also occur due to consumption of treated food (Berman et al. 2016) or as a consequence of gardening or of living next to agricultural areas (Galea et al. 2015, 2017; Linhart et al. 2019). CP and CPM possess moderate toxicity (Gül 2005; Alizadeh et al. 2018). However, it has been demonstrated that CP and CPM can inhibit acetylchloinesterease (Soto-Mancera et al. 2020), while exposure to CP has been correlated with neurological disorders (Qiao et al. 2002).

In mammals, CP and CPM are rapidly metabolized via a two-step metabolic pathway to produce the 3,5,6-trichloro-2-pyridinol (TCPy), either in free form or bound to glucuronic acid or sulfates as main metabolites, that are then secreted in urine (Barr and Needham 2002; Bicker et al. 2005). After CP oral administration, 70% of CP is excreted in the urine as TCPy with a half-time of 27 h (Nolan et al. 1984). Therefore, exposure of an individual to environmental contaminants such as CP and CPM can be determined by conducting biological monitoring of TCPy urinary levels. Hence, urinary TCPy has been used as a biomarker of exposure to CP and CPM in occupational (Fenske et al. 2013; Singleton et al. 2015) and general population (Aprea et al. 1999; Koch et al. 2001; Barr et al. 2005) frameworks.

There are no specific studies evaluating the occupational exposure or the exposure of people living near agricultural areas to CP and CPM in South Tyrol. The only study evaluating the exposure to CP in an Italian general population sample was conducted in 1999 (Aprea et al. 1999). Occupational exposure to CP was evaluated in Egyptian workers (Singleton et al. 2015) and in UK adults and children living near agricultural land (Galea et al. 2015).

Nevertheless, in a biological monitoring study, it should be accounted for that exposure to contaminants might be influenced by seasonal variation (Paglia et al. 2017). Weather conditions may influence exposure to pesticides. For instance, higher temperature can facilitate evaporation of spray droplets during treatment, but also other climate variables can considerably influence human exposure to pesticides (Otieno et al. 2013).

Therefore, the aim of this study was to obtain a longitudinal evaluation of the exposure to CP and CPM in agricultural workers in South Tyrol and in a residential group living in the same area, collecting samples in two different time periods: during the season of the pesticide treatment and in different season temporally far away from the treatment. The objective was first to evaluate CP and CPM exposure in the investigated area and second to assess whether such an exposure was associated with the pesticide treatment season.

To achieve this objective, first we developed the analytical procedure for the determination of TCPy concentrations in urine and then we designed a longitudinal study for biologically monitor TCPy in the urine of 28 farmers actively working in spraying pesticides and 43 residential people living in the same agricultural area.

Material and Methods

Study Design

The study was performed between May 14th, 2014, and March 16th, 2015 in South Tyrol, Italy. During the pesticide treatment season, between May 14th and May 29th, 2014, 28 farmers were enrolled in the study. Forty-three non-farmer residents from 8 municipalities were enrolled in the study between the 3rd and 13th of June 2014. A second sample per participant was taken from November 4th until December 19th, 2014 and from 21st January until 24th of February 2015 for farmers, and for the non-farmer residents. These two periods correspond to a season that is temporally far from the treatment season.

The farmer sample was selected from lists provided by the "Unione Agricoltori e Coltivatori Diretti Sudtirolesi". We selected farmers with > 4 hectares' agricultural surface, running > 70% of the pesticide treatment themselves, and whose home address was the same as that of their warehouse for the storage of plant protection products. Non-farmer residents were chosen based on maps from the "Ufficio frutti viticoltura". We identified houses bordering with agriculture fields. They should neither be family members of a farmer nor live in a house where a farmer lives. The final selection was made after home visit by the personnel of the Environmental Medicine Unit of the Healthcare System of the South Tyrol.

The study received ethical approval by the Ethical Committee of the Healthcare System of the Autonomous Province of Bozen/Bolzano. Participation was on a voluntary basis and all participants gave written informed consent in accordance with the Declaration of Helsinki. Each participant was asked to fill in a short questionnaire to collect demographic characteristics as well as information on factors that might influence TCPy concentration, such as: smoking (i.e., smoking less than vs more than 5 cigarettes per day); alcohol consumption (i.e., drinking less than vs more than 250 mL alcohol per day); or pharmacological therapy (i.e., taking any kind of drug during the last 30 days). The distribution of the number of study participants per sampling area and season is given in Table 1.

Study Area

South Tyrol has a population of about 536,000 inhabitants, mostly concentrated around the main urban centers.

Table 1 Characteristics of the study participants

Study participants	Farmers (*n* = 28)		Residents (*n* = 42)	
Season of sample collection	Non-treatment	Treatment	Non-treatment	Treatment
Demographic characteristics				
No. of females (%)	0	0	21 (49)	21 (49)
Age, median (min–max)	49 (23–63)	46 (23–63)	55 (5–73)	55 (5–76)
Exposures				
Smoking (%)	1 (4%)	1 (4%)	1 (2%)	1 (2%)
Alcohol consumption (%)	2 (7%)	1 (4%)	0 (0%)	1 (2%)
Drug treatment (%)	8 (29%)	5 (18%)	14 (33%)	14 (33%)

Notice that: Exposures were reported at the beginning of the study (see Methods); for two residents, only one measurement is available

In this study, an agricultural area of 240,535.4 hectares was selected, corresponding to approximately 30% of the total surface of the region. The agriculturally used area mainly consists of pastures (61%), permanent grassland (27%) and woody crops (10%). Farmers were enrolled from 5 municipalities (Kastelbell/Tschars, Latsch, Marling, Naturns, and Dorf Tirol) while non-farmer residents from 8 municipalities (Kastelbell/Tschars, Latsch, Naturns, Partschins, Plaus, Schlanders, Lana, and Tisens).

Chemicals

The analytical standards for 3,5,6 -trichloro-2-pyridinol purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). 3,5,6-trichloro[4,5,6-$^{13}C_3$] pyridin-2-ol was purchased from Cambridge Isotope Laboratories (Tewksbury, US) and was used as internal standard. Acetonitrile hypersolv CHROMANORM VWR (Radnor, USA), formic acid Optima LC–MS from Fischer Scientific (Waltham, USA), methanol LC–MS CHROMASOLV. Acetic Acid LC–MS grade and β-Glucuronidase from Helix pomatia was purchased from Sigma Aldrich (St. Louis, USA). LC–MS grade water was produced by Milli-Q Merck Millepore (Massachusetts, USA) water system using LC-pak Polisher cartridge containing C18 reverse phase silica.

Sample Preparation

The first urine in the morning was sampled by collecting a mid-stream urine sample from each participant in a 100 mL urine specimen cup and sent the same day at the Biobank of the Institute of Biomedicine located in Bozen/Bolzano.

After collection, samples were transferred from urine containers to a 10 mL tubes and centrifuged for 20 min at 1200 g. Supernatant was recovered, transferred in a new tube and aliquoted into twelve 200 μL aliquots and snap frozen and stored at − 80 °C until preparation for analysis. An aliquot was sent for to clinical pathology laboratory of the Hospital of Bozen/Bolzano for the determination of urinary creatinine concentrations using a colorimetric method based on a Jaffe reaction.

The incubation solution containing ß-Glucuronidase at the concentration of 534 units/mL, was prepared in 0.2 M acetate buffer and then was spiked with the internal standard (3,5,6-trichloro[4,5,6-13C3] pyridin-2-ol), to give the final concentration 13.3 ng/mL.

200 μL of samples, calibrators and quality controls (200 μL) were incubated for 17 h at 37 °C with 300 μL of the incubation solution to achieve hydrolysis of conjugated forms of TCPy into free TCPy.

A solid phase extraction (SPE) procedure was used for sample cleanup after incubation of samples. The method was modified from the method described by Olson et al. (Olsson et al. 2004). In brief, the Oasis SPE 96 well plate was first conditioned with 1 mL methanol, then with 1 mL 1% acetic acid. Incubated samples were added to the plate and passed through the plate with the aid of gravity. The samples were washed with 1 mL water, then 1 mL of 10% methanol to remove possible interfering components, then dried for 30 min using nitrogen with positive pressure manifold. Finally, the samples were eluted with 800 μL of methanol. The resulting eluate was then evaporated to dryness, and reconstituted in 200 μL 15% acetonitrile, and shaken for 20 min. The plate was then centrifuged for 15 min, and prior to analysis.

Liquid Chromatography Mass Spectrometry

The ultra-high performance liquid chromatography (UHPLC) part of the analytical system consisted of Agilent 1290 binary pump, a cooled autosampler and a column oven with a 10-port 2-position column-switching valve (Agilent Technologies, Santa Clara, CA, US).

Chromatographic separation was achieved using Waters ACQUITY UPLC BEH C18 column (130 Å, 1.7 μm, 2.1 mm X 100 mm, Waters Corporation, Milford, US). Mobile phase A consisted of water containing 0.1% formic acid (v/v) and mobile phase B consisted of acetonitrile containing 0.1%

formic acid (v/v). A gradient elution program was utilized with a total run time of 5 min with the following gradient profile, 0.00 min 15% B, 1.00 min 15% B, 3.0 min 95% B, 4.0 min 95% B, 4.1 min 15%B and 5.0 min 15% B. Column heater temperature was maintained at 60 °C and autosampler temperature at 8 °C.

Mass spectrometry detection was achieved with a QTRAP 6500 (Sciex, Framingham, MA, US) set in selected reaction monitoring (SRM) equipped with atmospheric pressure chemical ionization (APCI) source operated in negative ionization mode. The atmospheric chemical ionization temperature was optimized at 300 °C. Curtain gas and ion source gases 1 and 2 were set at 20, 80 and 80 psi, respectively. Nebulizer current was optimized at 3.0 µA. Collisional energy gas was set at 12 psi, and two SRM were monitored for 3,5,6 -trichloro-2-pyridinol and for 3,5,6-trichloro[4,5,6-$^{13}C_3$] pyridin-2-ol. The first transition was used for quantitation purposes, whereas the second one was used to confirm the presence of target compounds in the sample. Quantification SRM transition was m/z 195 > 35 and confirmation SRM transition was m/z 195 > 37 for 3,5,6 -trichloro-2-pyridinol. Quantification SRM transition was m/z 195 > 35 and confirmation SRM transition was m/z 195 > 37 for internal standard 3,5,6-trichloro[4,5,6-$^{13}C_3$] pyridin-2-ol.

Data recording was done with Analyst 1.6.3 and peak evaluation and integration were done with Multiquant 3.0.2 (Sciex, Framingham, MA, US).

Method Validation

In this work, we aimed at monitoring longitudinal exposure to CP and CPM, therefore we first developed a specific and sensitive LC–MS/MS method targeting urinary TCPy and then and then we validated such a method following the European Medicines Agency Guidelines of Bioanalytical Validation. Stock solution of TCPy (SS1) was prepared by weighing 2 mg of 3,5,6-Trichloro-2-pyridinol that were then transferred to a 10 mL volumetric flask and dissolved in acetonitrile to give a solution at concentration of 200 µg/mL. Intermediate stock solution (SS2) was prepared by one-step dilution of SS1 with 50% acetonitrile, to give the concentration of 20.0 µg/mL. Working solutions for the preparation of calibration standards were made with serial dilution from SS2 in the concentration of 5, 25, 50, 200, 400, 1000, 2500, 5000 ng/mL respectively. Same procedure was done for quality controls (QCs) by serial dilution of SS2 to final concentrations of 75, 375, 1500, 3750 ng/mL.

Calibrators and QCs were prepared by spiking blank urine to give the final concentration of 0.1, 0.5, 1, 4, 8, 20, 50, 100 ng/mL for calibrators and 0.1, 0.5, 1.5, 7.5, 30, 75 ng/mL QCs.

Calibrators were prepared freshly for each assay and extracted along with urine samples and quality controls. The added volume was always less than 4% of total volume of the samples, so that the integrity of the matrix was maintained.

The lower lowest limit of quantification (LLOQ) was defined as the lowest point on the calibration curve providing a signal to noise ratio of 10 and a precision of 20% and accuracy ± 20%.

Selectivity of the analytical method was assessed by analysing extracted blank samples to investigate possible interference and compared to extracted LLOQ sample.

The linearity of the method was evaluated by analysing three different sets of calibration curves, with the range of 0.5–100 ng/mL. Intra-day accuracy and precision were evaluated by analysing on the same day six replicates for each QCs at different concentration levels of TCPy. Inter-day accuracy and precision were evaluated by comparing replicates of QCs analysed in different batches on different days.

Matrix effect was evaluated using five different sources of a blank urine to generate five different calibration curves as recommended by Matuszewski et al. (Matuszewski et al. 2003). Then, comparison of the slopes obtained in different urines was used to estimate the matrix effect.

Bioanalytical guidelines suggest a minimum of 7% of all samples have to be re-analyzed showing that 67% of these repeats must have a percent difference less than 20%. We validated our results by re-analysis of 16 urine real samples (10%).

The reproducibility of the method was further validated by analyzing the same urine samples independently in a laboratory specialized in environmental exposure measurements (Institut und Poliklinik für Arbeits-, Sozial- und Umweltmedizin, Schillerstr. Erlangen. Germany). The method used in the independent laboratory was based on gas chromatography mass spectrometry (GC–MS).

Statistical Analysis

The sampling validation was carried on evaluating intra-day precision and accuracy, quantified as the relative standard deviation percent (RSD%), that is, the standard deviation divided by the absolute value of the mean, and the relative error percentage (RE%) of replicates, that is, the ratio between the replicate and the true value. RSD% was calculated for the calibration curves too. Correlation and concordance of the two TCPy measurements obtained from our laboratory and the independent one were estimated using the Pearson's correlation coefficient and the Lin's concordance correlation coefficient(Lin 1989), respectively. In detail, the Pearson's coefficient allowed to assess how closely related the two sets of measurements were, assuming a linear relationship between them with a baseline not equal to zero; whereas the Lin's coefficient allowed to assess the agreement

between the two sets assuming a linear relationship with a baseline equal to 0 and a slope equal to 1.

Median values were provided given the skewed distributions.

In the longitudinal investigation, we defined outliers as values located outside 1.5 times the interquartile range, within each group including all samples. After outliers' exclusion, we considered only participants with both measurements available. Given the small sample size and the asymmetric sample distribution, between-group comparisons were tested using the Wilcoxon rank sum test at a significance level of 0.05. The effect of additional exposures (smoking status, alcohol consumption, and drugs therapy) on TCPy levels was evaluated using the same test, comparing exposed and not exposed participants within each season.

Results and Discussion

Validation of the Analytical Method

Traditionally the determination of CP and CPM and their metabolites in different matrices, including biological specimens, have been performed using GC–MS and LC–MS (Koch et al. 2001; Olsson et al. 2004; Curwin et al. 2010; Martínez-Domínguez et al. 2014; Radford et al. 2014; Soares et al. 2019).

Intra-day precision and accuracy were determined by analyzing quality control (QC) samples replicates at five different concentrations of TCPy in urine during the same day. RSD% ranged from 2.0 to 7.5 proving that all QCs were within the 15% RSD limit for precision (Table 2). RE% ranged from -5.5 to 0.4 demonstrating that intra-day accuracy was the 15% for all QCs (Table 2).

Inter day precision and accuracy were determined by analyzing QC samples replicates at 5 different concentrations of TCPy in urine in three different days. RSD% ranged from 2.6 to 9.5, while RE% ranged from -5.5 to 0.4, demonstrating that inter-day accuracy and precision were both within the 15% for all QCs (Table 2).

The recovery was determined by analyzing TCPy in six replicates at three different concentration levels by comparing mean peak area of urine spiked before and after sample preparation. The recovery of the TCPy ranged from 68 to 70% (Table 3).

In addition, further experiments were performed to assess the matrix effect. Five different sources of blank urines were used to generate five different calibration curves. Comparing the slopes of these calibration curves in five different sources of a urine provides an estimation of matrix effect. The RSD% obtained for the five slopes was 4.6% suggesting the absence of any significant matrix effect on quantification.

A total of 16 (10%) samples were re-analyzed in this study with 15 (94%) of them meeting the requirement of 20% or less absolute difference. The single re-analysis sample that failed had a percentage difference of 24%.

The reproducibility of the method was further validated by re-analyzing all samples by an independent laboratory

Table 2 Intra- and inter-day accuracy and precision of TCPy replicates at five different concentrations in urine during the same day

QC (ng/mL)		Intra-day ($n=6$)		Inter-day ($n=6$)	
		Precision (%RSD)	Accuracy (%RE)	Precision (%RSD)	Accuracy (%RE)
LLOQ	(0.5 ng/mL)	7.5	− 1.4	8.9	1.4
QC-LOW	(1.5 ng/mL)	6.3	− 1.1	9.5	5.6
QC-LM	(7.5 ng/mL)	3.9	0.4	7.1	2.4
QC-HM	(30.5 ng/mL)	3.0	− 2.3	5.1	2.2
HQC-HIGH	(75 ng/mL)	2.0	− 5.5	2.6	6.5

Abbreviations: *%RSD*=Relative standard deviation %; *%RE*=Relative error %; *LLOQ*=Lower limit of quantification; *QC*=Quality Control; *HQC*=High quality control

Table 3 Estimation of the recovery of urinary TCPy

QC (ng/mL)		Spiked before sample prep		Spiked after sample prep		Recovery
		Signal Area	RSD%	Signal Area	RSD%	
QC-LM	(7.5 ng/mL)	6220	13%	8840	3%	70%
QC-HM	(30.5 ng/mL)	22,800	11%	33,600	4%	68%
HQC-HIGH	(75 ng/mL)	55,400	9%	81,400	5%	68%

Abbreviations: *%RSD*=Relative standard deviation %

using a previously validated method for the analysis of TCPy based on GC–MS (Koch et al. 2001).

The two absolute measurements of the TCPy show high correlation (Pearson´s coefficient = 0.95) and good concordance (Lin´s coefficient = 0.82 with a 95% confidence interval from 0.79 to 0.85) (Figure S1).

The comparison indicated a slight bias towards the outside laboratory producing higher absolute values but was well within what could be expected.

Monitoring Exposure to Chlorpyrifos and Chlorpyrifos-Methyl in Farmers and Non-Farmer Residents

Summary characteristics of study participants are reported in Table 1. Urinary TCPy levels were monitored in 28 farmers actively working in spraying pesticides and in 42 residential people living in the same agricultural area in two periods, during the treatment season and in a second period temporally far from the treatment season (Table 1).

For the statistical analysis we first considered only participants with two longitudinal measurements obtained in

both periods resulting in a dataset of 25 pairs of exposed farmers and 41 pairs of exposed residential people.

A total of 6 outliers were identified, two among farmers and four among residents. Outliers were removed yielding to a final dataset containing 23 pairs of farmers and 37 pairs of residents (Table 4).

During the spraying season urinary concentration of TCPy in farmers ranged from 3.67 to 13.59 µg/g of creatinine. A similar study, reported urinary TCPy levels ranging from 16.4 to 30.1 µg/g of creatinine in individuals spraying CP and CPM (Singleton et al. 2015), suggesting lower exposure to CP and CPM in the present study. As expected, urine TCPy levels of farmers directly exposed to CP and CPM during the treatment season were significantly higher when compared with levels obtained in a second period far from the treatment (Table 4 and Fig. 1).

A similar result was obtained for the 37 exposed residential people living in the same agricultural area. Indeed, their urinary levels of TCPy during the treatment season ranged from 1.30 to 13.98 µg/g of creatinine. The impact of spraying season on people living near areas with pesticide-intensive agriculture was remarkable, since the urinary concentration of TCPy in residents showed no

Table 4 Summary statistics of TCPy after removing outliers

Subjects	Farmers		Non-farmers	
Season	Non-treatment	Treatment	Non-treatment	Treatment
TCPy distribution (ug / g creatinine)				
Mean (SD)	2.76 (1.08)	7.73 (3.06)	4.3 (2.57)	6.87 (3.65)
Median (min–max)	2.54 (1.16–4.96)	6.8 (3.67–13.59)	3.22 (0.92–11.84)	6.73 (1.30–13.98)
Geometric mean	2.56	7.16	3.68	5.83
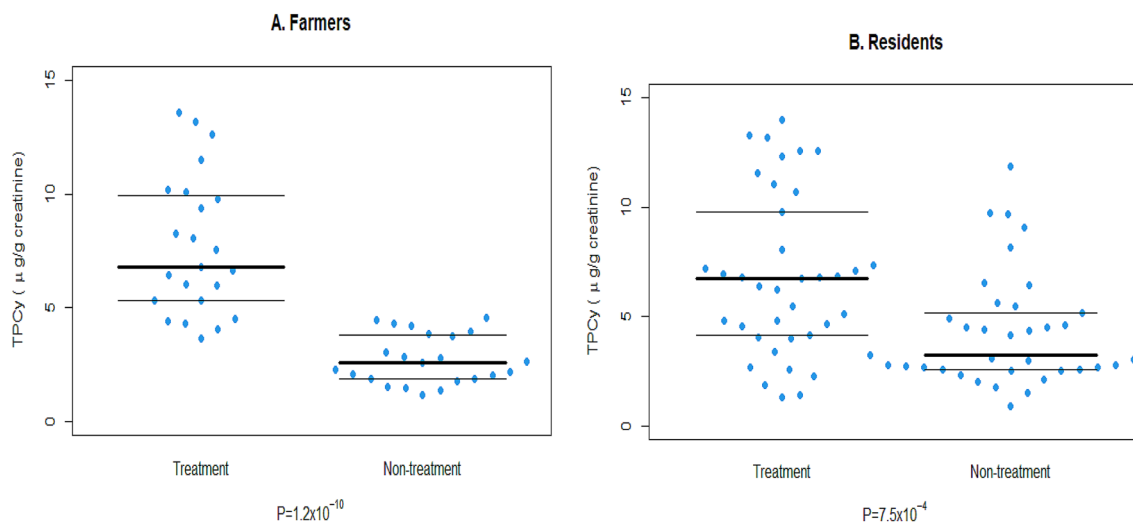
Fig. 1 TCPy level distributions comparison by season: 1 = Treatment; 2 = Non-treatment. **A** Farmers. **B** Residents. P is the p-value of the Wilcoxon test

significant difference (*p*-value = 0.332) with respect to those observed in the urine of farmers recorded in the same period (Table 4 and Fig. 1).

These data suggest that exposure to CP and CPM was similar for farmers actively spraying pesticides and residents living near to the sprayed areas.

The FAO/WHO organization suggests for both CP and CPM an acceptable daily intake (ADI) of 10 µg per kg bodyweight per day. Urinary TCPy concentration of 2.8 µg/l urine corresponds to a daily intake of approximately 5 µg CP/CPM (Koch et al. 2001).

Therefore, the reported levels of TCPy resulted in a daily intake of CP and CPM well below the recommended ADI according to FAO/WHO.

It is interesting to note that the exposure to CP and CPM was associated with higher level of TCPy in both groups, but such a higher level was not proportional to the basal level recorded outside the treatment season (Table 4 and Fig. 1). Out of the treatment season, farmers and residents showed a significantly different distribution of TCPy (p-value = 6×10^{-3}) (Fig. 1) suggesting the presence of unmeasured factors associated with other sources of exposure.

Alcohol and tobacco consumption can modify metabolic and physiological processes and impact both metabolism and pharmacokinetics of xenobiotics such as CP and CPM, and might influence the TCPy concentration (Lee et al. 2011). However, repeating the analyses by accounting for the additional risk factors collected by this study (smoking status; alcohol consumption, and drugs therapy) did not identify any possible explanation for the different TCPy levels either by groups of participants (farmers vs residents), season, or combination of both (Figure S2).

We suggest that other sources of exposure that were not considered in this study might play a role in the exposure to CP and CPM. In fact, the higher levels of TCPy observed during the non-treatment season might be due to several unexplored factors such as a vegetarian diet (Berman et al. 2016). Another explanation could be given by the fact that farmers were not selected based on the area they live, and they might live far away from the sprayed zones resulting in less exposure during the non-treatments season. Unfortunately, this is a limitation of this study since neither diet information nor information related to the residential address of the farmers were collected in the course of our study.

Finally, the geometric mean of TCPy detected far from the spraying season in all subjects (farmers and non-farmers) was of 3.6 µg/g of creatinine. This value is in agreement with data from the literature recorded in different countries (Aprea et al. 1999; Koch et al. 2001; Barr et al. 2005; Roca et al. 2014; Berman et al. 2016) (Table S1). Therefore, the overall exposure to CP and CPM in the portion of South Tyrol considered in this study comparable with that other countries during a period far from the spraying season.

Conclusion

The organophosphorus pesticides CP and CPM are known to inhibit acetylchloinesterease (Soto-Mancera et al. 2020) and to be correlated with neurological disorders (Qiao et al. 2002). CP and CPM has been used in in South Tyrol to prevent "apple proliferation" and both farmers and general population living in rural areas have been potentially exposed to these pesticides. To assess the levels of TCPy following exposure to CP and CPM in farmers and general population individuals, we conducted a longitudinal study by measuring the TCPy metabolite on urine samples of farmers and residential people living close to agricultural fields. Samples were collected during the season of spraying CP and CPM treatment and in a second season far from the treatment one. A LC–MS/MS method targeting urinary TCPy was first developed and validated and then applied for monitoring exposure to organophosphorus pesticides CP and CPM.

We observed slightly higher level of TCPy in both farmers and general population living in rural area in Val Venosta during the treatment seasons, suggesting that the impact of spraying season might be similar on farmers and people living near agricultural areas. The reported levels of TCPy, however, resulted in a daily intake of CP and CPM well below the ADI recommended by FAO/WHO.

Furthermore, during the non-treatment season, non-farmers living in rural area had the highest value among the two groups investigated, suggesting that in the general population TCPy might result in altered levels because of other unmeasured sources of exposure not considered in this study, such as diet. Hence, we suggest that other sources of exposure not considered in this study might play a significant role in exposure to CP and CPM. Comparing our data with scientific literature regarding the recorded values in a period far from the treatment season suggest that exposure to CP and CPM in general population is similar.

BS and SS performed the experiment and contributed to writing the manuscript. CC MW CP and LW designed the study and contributed to writing the manuscript. The authors thank the Department of Innovation, Research and University of the Autonomous Province of Bozen/Bolzano for covering the open access publication cost.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

Ethical Approval Participation was on a voluntary basis and all participants gave written informed consent in accordance with the Declaration of Helsinki. The study received ethical approval by the Ethical Committee of the Healthcare System of the Autonomous Province of Bozen/Bolzano.

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