



# Evaluation of Potential DNA-Damaging Effects of Nitenpyram and Imidacloprid in Human U937-Cells Using a New Statistical Approach to Analyse Comet Data

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## Abstract

Even if the two neonicotinoids nitenpyram and imidacloprid have been considered safe for humans, their potential genotoxicity still remains a matter of discussion. The DNA-damaging effects of these two compounds were therefore evaluated in a lymphoma cell line of human origin (U-937) using the comet assay after 3-h exposure to up to 50  $\mu\text{M}$ , with or without metabolic activation using S9 from human liver. The comet data were analysed using a traditional one-way ANOVA after pooling the data on cellular level, and a new alternative approach we have called Uppsala Comet Data Analysis Strategy (UCDAS). UCDAS is a proportional odds model tailored to continuous outcomes, taking the number of pooled cultures, slides and cells into consideration in the same analysis. To the best of our knowledge, the UCDAS approach when analysing comet data has never been presented before. Without metabolic activation, no increase in DNA damage was observed in the neonicotinoide-exposed cells. Nitenpyram was also without DNA-damaging effects when S9 was added. However, in the presence of S9, imidacloprid was found to increase the level of DNA damage. Whereas the ANOVA showed an increase ( $P < 0.001$ ) both at 5 and 50  $\mu\text{M}$ , UCDAS showed an increase only at the lowest concentration ( $P < 0.001$ ). Based on these findings, the two neonicotinoids seem to be of little concern when it comes to their potential genotoxicity. However, since the U-937 cells were rather resistant to our positive controls, they may not be the best cells to use when evaluating potential genotoxicity of chemicals.

**Keywords** Neonicotinoids · Imidacloprid · Nitenpyram · In vitro comet assay · DNA damage · Metabolic bioactivation · Statistical analysis of comet data

## Introduction

Neonicotinoids are nicotine derivatives used as insecticides and in veterinary parasite remedies (Calderon-Segura et al. 2012; Schenker et al. 2003). Apart from an occupational exposure, humans can be exposed by consuming food containing residues of neonicotinoids (Cimino et al. 2017). Being agonists to the nicotinic acetylcholine receptor (nAChR), neonicotinoids are neurotoxic (Bianchi et al. 2015), but since they have much greater affinity to invertebrate receptor, the  $\text{LD}_{50}$  of these compounds is about 450

times higher in mammals than in insects (Tomizawa and Casida 2005). Because of their much lower acute toxicity in mammals, neonicotinoids have been considered safe for use by humans to combat pests in plants and animals. However, their potential genotoxicity is still a matter of discussion.

The use of neonicotinoids in insecticides has grown rapidly since their introduction in 1991 (Calderon-Segura et al. 2012). In 2008, they accounted for 24% of the insecticide market share (Jeschke et al. 2011). Imidacloprid had the highest sale value of all insecticides in 2009 but has since then been banned in the European Union (EU) due to its toxic effects in honeybees (Jeschke et al. 2011). As recent as in April 2018, the member states of EU decided to accept the recommendation proposed by the European Commission to expand the previous ban on neonicotinoide use (Union 2018). The extended ban restricts and prohibits the use of the three most commonly used neonicotinoids: imidacloprid, clothianidin and thiamethoxam within open field agricultural

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industry, but they can still be used in enclosed greenhouses. Since nitenpyram has a lower acute toxicity in honeybees than imidacloprid, it was still used as an insecticide after the latter compound was banned. When used as veterinary drugs, the story of the two neonicotinoids is a little bit different. While both are approved as parasite treatments for pets by the Federal Drug Agency (FDA) in the US, only imidacloprid has been approved for that purpose by the European Medicines Agency (EMA) (Iwasa et al. 2004; Tomizawa and Casida 2005).

While the toxicity of neonicotinoids in honeybees has been investigated extensively, studies on material of human origin seem sparse, especially when it comes to nitenpyram. Whereas there are some in vitro studies of the cytotoxicity of imidacloprid in human cells (Al-Sarar et al. 2015; Bianchi et al. 2015; Calderon-Segura et al. 2012; Senyildiz et al. 2018; Zeljezic et al. 2016)—no similar studies of nitenpyram were found in our literature search. A similar picture emerges when it comes to genotoxicity studies of imidacloprid and nitenpyram. There are some in vitro studies on the potential genotoxicity of imidacloprid in human cells using either HepG2-cells, peripheral lymphocytes or SHSY-5Y cells, showing genotoxic effects at  $\mu\text{M}$  concentrations using the comet assay or the micronucleus assay (Bianchi et al. 2015; Calderon-Segura et al. 2012; Costa et al. 2009; Feng et al. 2005; Senyildiz et al. 2018; Zeljezic et al. 2017).

Using the alkaline version of the comet assay for DNA-damaging effects, one of the major aims of the present study was to evaluate and compare the potential DNA-damaging effects of imidacloprid and nitenpyram in a histiocytic human lymphoma (U-937) cell line (Sundstrom and Nilsson 1976), not previously used to assess the potential genotoxicity of neonicotinoids. However, since both of these neonicotinoids contains a 2-chloropyridine moiety (Fig. 1), another major objective was to evaluate if these two compounds are bioactivated when a S9-fraction from human liver is added during the exposure. To the best of our knowledge, such studies have not been published yet. However, it has been shown that 2-chloropyridine may induce DNA damage in the presence of S9 (Chlopkiewicz et al. 1993), possibly triggered by the interaction between 2-chloropyridine and one of its N-oxidized metabolites (Anuszewska and Koziorowska 1995).

The third major objective of the present study was to address the issue of statistical analysis of comet data. The comet assay was introduced more than 30 years ago (Singh et al. 1988), but it still appears as if there is no consensus on how to handle the statistical analysis of this type of data. In the present paper, we propose a new statistical approach to evaluate comet data: a so-called proportional odds model tailored to continuous outcomes which we have called UCDAS.

## Materials and Methods

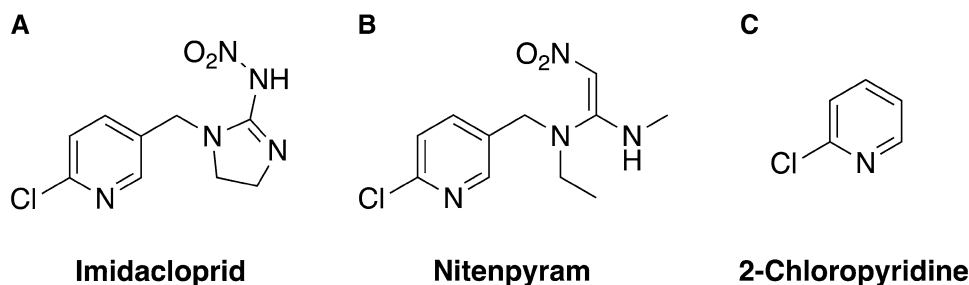
### Chemicals

Imidacloprid (CAS Reg. No. 138261-41-3), nitenpyram (CAS Reg. No. 150824-47-8), chlorambucil (CAS Reg. No. 305-03-3) and cyclophosphamide (CAS Reg. No. 6055-19-2) were all purchased from Merck (Darmstadt, Germany). Chlorambucil was used as the positive control in the Comet assay experiments without S9; cyclophosphamide was used as the positive control in the experiments with S9. Culture medium was used as vehicle (i.e. as the negative control). Unless indicated otherwise, these and all other chemicals and reagents used were of analytical grade, and double-distilled water was used throughout the experiments.

### Cells, Culture Medium and Metabolic Activation System

The human histiocytic lymphoma (U-937) cells (generously supplied to us by Dr. Robert Burman) were cultivated in Gibco™ RPMI 1640 medium (Fisher Scientific, UK) complemented with 10% foetal bovine serum (FBS; Biological Industries, Israel), 1% Gibco™ Pen Strep (10,000 U penicillin/ml and 10,000  $\mu\text{g}/\text{ml}$  streptomycin; Fisher Scientific, UK), and 1% sodium pyruvate (Fisher Scientific, UK), hereafter referred to as the growth medium. After an ampoule of frozen cells had been carefully thawed, the cells were maintained in an atmosphere with 5%  $\text{CO}_2$  until the experiments were performed on cells in suspension on cultivation day 6 (using the same batch of cells for all experiments). The metabolic activation systems used was based on S9-fractions from human livers (Merck, Germany). Following the

**Fig. 1** Structural formulas of **a** imidacloprid, **b** nitenpyram and **c** 2-chloropyridine



protocol of Sasaki et al. (2007), the human S9-mix was freshly prepared using 5% human S9, 4 mM MgCl<sub>2</sub>, 16.5 mM KCL, 2.5 mM glucose-6-phosphate, 50 mM Na<sub>2</sub>HPO<sub>4</sub> and 2 mM NADPH (the latter co-factor was added just before use).

### Exposure Conditions

The DNA-damaging effects of imidacloprid and nitenpyram after 3-h exposure were evaluated using 0.5 μM as the lowest concentration and a 100 times higher concentration as the highest concentration without metabolic activation system, and at 5 or 50 μM with S9. Since the highest concentration used in the Comet assay could be dissolved directly in the medium (also when making the stock solutions), growth medium was used as the negative control. Two different positive controls were used: chlorambucil (3 mM) without S9 and cyclophosphamide (0.1 mM) with S9. Exposures without S9: Aliquots of U-937 cell suspensions (cells plus growth medium) were added to test tubes (approximately 800,000 cells per tube) together with 10 μl stock solutions of either neonicotinoids, positive control or medium, adjusting the final volume to 1 ml with medium. Exposures with S9: Aliquots of cell suspension and 10 μl stock solutions of compounds were added to test tubes without S9, but in this case 160 μl S9-mix (50 μl S9, 100 μl medium and 10 μl NADPH) were also added, adjusting the final volume to 1 ml with medium.

Before and after all exposures, cell viability was checked using the trypan blue exclusion technique which assumes that non-viable cells are stained blue. A relative viability (relative to the negative controls) of at least 70% was used as a cut-off for evaluating the level of DNA damage in the Comet assay (Tice et al. 2000).

### Evaluation of DNA Damage Using the Comet Assay

Our standard procedure for the alkaline version (pH > 13) of the Comet assay is based on a slightly modified protocol of Singh et al. (1988), previously described in great detail (Andersson et al. 2003; Demma et al. 2009; Vaghef et al. 1996) with some minor modifications. Briefly, 60 μl of a mixture of 30 μl of cell suspension in growth medium (approximately  $1 \times 10^5$  cells/ml) and 210 μl 0.6% low-melting point agarose (International Biotechnologies, US) was layered on top of an ordinary microscope slide pre-coated with a layer of 0.6% low-melting point agarose. To avoid slipping gels and biased evaluation of DNA damage, the slides had been engraved about 1 mm from each of its four edges and coded, respectively. The agarose was allowed to set on ice for 15 min before the cells were lysed at 4 °C for 1 h in a freshly prepared lysis solution (2.5 mM NaCl, 100 mM Na<sub>2</sub>EDTA, 10 mM Tris, NaOH to pH 10, 1%

Triton X-100 and 10% DMSO). After lysis, the cells were immediately transferred to an electrophoresis unit (Sigma, Horizontal, Dual mode) in which the supercoiled DNA was relaxed in the electrophoresis buffer (0.3 mM NaOH and 1 mM Na<sub>2</sub>-EDTA, pH > 13) at 4 °C for 40 min. Immediately after the electrophoresis (for 10 min at 25 V and 300 mA), the slides were neutralized with 0.4 M Tris-HCL, pH 7.5, dried at room temperature in a hood, and stored in a sealed box until the day of image analysis.

After dipping the slides in neutralization buffer to moisten the dried gels, propidium iodide (30 μl, 0.15 mM) stained nucleoids were examined in a fluorescence microscope (Olympus BX60F-3, Olympus Optical, Japan). Digitized images (50 randomly selected cells per slide; employing three slides per treatment and culture) were scored using an ATV FireWire camera (Stingray; Allied Technologies, Germany) and the software Comet Assay IV (Perspective Instruments, UK).

### Statistical Analysis of Comet Data

After decoding the slides, the mean tail intensities (TI; TDNA; percentage of DNA in the tail) from each individual experiment and treatment (usually at least three cultures/treatment) were used as an indicator of the level of DNA damage. The association between total DNA damage and the different treatments was analysed using both (i) a traditional one-way ANOVA followed by a Tukey post hoc test on pooled cell data using the software Statistica (TIBCO Software Inc., USA), and (ii) a proportional odds model tailored to continuous outcomes which we have named UCDAS using the statistical program R version 3.5.1. For the latter approach, a working independence model was first fit by assuming independent observations. Since UCDAS does not consider the possible dependencies in the data induced by the experimental design, standard errors were adjusted using a cluster robust covariance matrix where the clusters were defined as cells within slides nested within treatment and experiment. Post hoc treatment comparisons were adjusted for the number of comparisons made to control the family wise error rate at 5%.

### Results

When tested in concentrations up to 50 μM, neither imidacloprid nor nitenpyram were found to increase the level of DNA damage in the U-937 cells after 3 h of exposure without metabolic activation (Table 1). When the cells were exposed for 3 h in the presence of human S9, imidacloprid increased the level of DNA damage both at 5 and 50 μM according to the ANOVA analysis, but only at 5 μM according to the UCDAS analysis (Table 2). As

**Table 1** Effects of two different neonicotinoids on the DNA migration in human U937-cells after 3 h of exposure without metabolic activation

Exposure	Concentration ( $\mu\text{M}$ )	Tail intensity (% TDNA)		Viability (%)	Number of cultures/slides/cells
		Median $\pm$ SE	Mean $\pm$ SEM		
Growth medium	0	0.5 $\pm$ 0.1	2.6 $\pm$ 0.2	$\geq$ 95	6/18/900
Chlorambucil	$3 \times 10^3$	14.8 $\pm$ 0.2***	15.2 $\pm$ 0.4†††	$\geq$ 85	6/18/900
Imidacloprid	0.5	0.4 $\pm$ 0.3	2.6 $\pm$ 0.3	$\geq$ 95	3/9/450
	5	0.3 $\pm$ 0.2	2.3 $\pm$ 0.2	$\geq$ 95	3/9/450
	20	0.5 $\pm$ 0.2	2.5 $\pm$ 0.2	$\geq$ 95	3/9/450
	35	0.4 $\pm$ 0.2	2.3 $\pm$ 0.2	$\geq$ 95	3/9/450
	50	0.4 $\pm$ 0.1	2.3 $\pm$ 0.2	$\geq$ 95	3/9/450
Nitenpyram	0.5	0.6 $\pm$ 0.2	2.9 $\pm$ 0.2	$\geq$ 95	3/9/450
	5	0.4 $\pm$ 0.1	2.5 $\pm$ 0.2	$\geq$ 95	3/9/450
	20	0.5 $\pm$ 0.2	2.9 $\pm$ 0.2	$\geq$ 95	3/9/450
	35	0.7 $\pm$ 0.2	2.7 $\pm$ 0.2	$\geq$ 95	3/9/450
	50	1.1 $\pm$ 0.2	3.4 $\pm$ 0.2	$\geq$ 95	3/9/450

Using a traditional protocol for the Comet assay, the cells were subjected to alkaline single-cell gel electrophoresis for 10 min after 3 h of exposure without metabolic activation to different concentrations of imidacloprid or nitenpyram. Chlorambucil was used as the positive control, and growth medium (vehicle) as the negative control. The tail intensity was used as the indicator of DNA damage. The medians ( $\pm$  standard error) and means ( $\pm$  standard error of mean) presented in the table were calculated after pooling the data from three or six independent experiments ( $n=450$  or  $900$ ). Two different methods were used for the statistical analysis: (i) a new proportional odds model tailored to continuous outcomes (UCDAS) and (ii) a traditional one-way ANOVA with Tukey post hoc test

\*\*\* $P < 0.001$  (UCDAS)—in comparison to vehicle control

††† $P < 0.001$  (ANOVA)—in comparison to vehicle control

also indicated in Table 2, the increase in DNA damage observed after the imidacloprid exposure showed no dose response, and even if there was a slight increase at 50  $\mu\text{M}$ , nitenpyram was judged to be without DNA-damaging effects also in the presence of S9 ( $P > 0.05$  both in the

ANOVA and UCDAS analyses). For all treatments (including the positive controls chlorambucil and cyclophosphamide which both induced pronounced DNA damage), the viability was always found to be well above the cut-off limit of 70% (Tables 1 and 2).

**Table 2** Effects of two different neonicotinoids on the DNA migration in human U937-cells after 3 h of exposure in the presence of a metabolic activation system

Exposure	Concentration ( $\mu\text{M}$ )	Tail intensity (% TDNA)		Viability (%)	Number of cultures/slides/cells
		Median $\pm$ SE	Mean $\pm$ SEM		
Growth medium + human S9	0	1.4 $\pm$ 0.3	4.8 $\pm$ 0.1	$\geq$ 90	3/9/450
Cyclophosphamide + human S9	100	3.8 $\pm$ 0.5***	8.6 $\pm$ 0.2†††	$\geq$ 85	3/9/450
Imidacloprid + human S9	5	2.8 $\pm$ 0.5***	7.3 $\pm$ 0.1†††	$\geq$ 90	3/9/450
	50	2.2 $\pm$ 0.4	6.8 $\pm$ 0.1†††	$\geq$ 90	3/9/450
Nitenpyram + human S9	5	1.4 $\pm$ 0.4	6.2 $\pm$ 0.1	$\geq$ 90	3/9/450
	50	1.2 $\pm$ 0.4	6.0 $\pm$ 0.1	$\geq$ 90	3/9/450

Using a traditional protocol for the Comet assay, the cells were subjected to alkaline single-cell gel electrophoresis for 10 min after 3 h of exposure to two different concentrations of imidacloprid or nitenpyram in the presence of S9 from human liver. Cyclophosphamide was used as the positive control, and growth medium (vehicle) as the negative control. The tail intensity was used as the indicator of DNA damage. The medians ( $\pm$  standard error) and means ( $\pm$  standard error of mean) presented in the table were calculated after pooling the data from three independent experiments ( $n=450$ ). Two different methods were used for the statistical analysis: (i) a new proportional odds model tailored to continuous outcomes (UCDAS) and (ii) a traditional one-way ANOVA with Tukey post hoc test

\*\*\* $P < 0.001$  (UCDAS)—in comparison to vehicle control

††† $P < 0.001$  (ANOVA)—in comparison to vehicle control

## Discussion

In the present study, the potential DNA-damaging effects of the two neonicotinoids imidacloprid and nitenpyram were evaluated in human U-937 lymphoma cells after 3-h exposure to up to 50  $\mu\text{M}$ , with or without a metabolic activation system based on S9 from human liver. The DNA damage was evaluated using a traditional version of the comet assay under alkaline conditions using two different statistical methods to analyse the data: (i) a traditional one-way ANOVA, and (ii) a new approach using a proportional odds model tailored to continuous outcomes (UCDAS). Neither neonicotinoid was found to increase the level of DNA damage in the absence of metabolic activation system. Although imidacloprid, but not nitenpyram, increased the DNA migration in the presence of S9, it did not follow a dose–response relationship since a higher level of damage was observed at 5  $\mu\text{M}$  than at 50  $\mu\text{M}$ . Taken together, our results indicate that the two neonicotinoids seem to be of little concern when it comes to their potential DNA-damaging effects, at least under the experimental conditions used in the present study employing human U-937 lymphoma cells.

When evaluating the genotoxicity of compounds, one shall also monitor a potential concomitant increase in cytotoxicity and/or decrease in cell viability. In the present study, using the trypan blue exclusion technique, the viability was constantly found to be at least 90% for the two neonicotinoids (Tables 1 and 2), i.e. well above 70% which usually is used as the cut-off in the comet assay. The percentage of so-called clouds (comets without a distinct comet head, also called hedgehogs), which we have used as a proxy for cytotoxicity in previous papers (Andersson et al. 2003; Bergqvist et al. 1998), was also extremely low for all treatments involving the neonicotinoids (results not shown). Moreover, in pilot studies performed before selecting the concentrations of the neonicotinoids for the Comet assay using the Pierce™ LDH Cytotoxicity Assay Kit (Thermo Scientific, UK), no cytotoxicity was observed for the two neonicotinoids at any of the concentrations tested, where the highest concentrations corresponded to the maximum solubility of the compounds using 1% DMSO as the solvent (results not shown).

It can be argued that we should have tested higher concentrations of the neonicotinoids by dissolving them in DMSO even if we did not see any cytotoxic effects in the pilot studies, but it shall be noted that the lowest concentration of neonicotinoid used without S9 in our comet assays (0.5  $\mu\text{M}$ ) is a reasonable biologically relevant concentration, at least according to Zeljezic et al. (2016) who estimated that the concentration of imidacloprid in human serum in a male human weighing 65 kg would be

around 0.1  $\mu\text{g}/\text{ml}$  (0.4  $\mu\text{M}$ ) at an estimated acceptable daily intake. The highest concentration used in the evaluation of potential DNA damage was set to 50  $\mu\text{M}$  as it was considered well above realistic serum levels and could be achieved from stock solutions using on growth medium as the solvent.

Reduced cell viability has been reported in a study on human peripheral lymphocytes exposed in vitro to commercial formulations of insecticides containing imidacloprid or other neonicotinoids after 2-h exposure to much higher concentrations than was used in the present study (Calderon-Segura et al. 2012). However, whether this was an effect of the neonicotinoids themselves or co-formulants in the commercial insecticides remains a matter of speculation. Calderon-Segura et al. (2012) also reported a significant increase in DNA damage in peripheral lymphocytes exposed to non-toxic concentrations of commercial insecticides containing neonicotinoids. Another commercial insecticide containing imidacloprid was used in a study on human hepatocellular carcinoma (HepG2) cells where increased cytotoxicity and increased level of DNA damage were observed after 24-h exposure (Bianchi et al. 2015). The cytotoxicity of imidacloprid in vitro has also been investigated in Chinese hamster ovary cells (Al-Sarar et al. 2015) and in a cell line based on gill cells from *Paralichthys olivaceus* (Su et al. 2007).

In a more recent study (Senyildiz et al. 2018) investigating the cytotoxic and DNA-damaging effects of imidacloprid and four other neonicotinoids in human neuroblastoma (SH-SY5Y) cells and HepG2-cells, significant DNA damage was observed in the imidacloprid-treated SH-SY5Y cells after 24- and 48-h exposures to 500  $\mu\text{M}$ . No increase in DNA damage was observed in the imidacloprid exposed HepG2-cells. To obtain such a high concentration of imidacloprid, the substance was dissolved in DMSO. Again, rather high concentrations were required to induce cytotoxicity. The  $\text{IC}_{50}$  for imidacloprid in the SH-SY5Y-cells was reported to be 2.23 to 4 mM, but no increase in cytotoxicity could be observed in the HepG2-cells at the highest concentration tested (4 mM). A general impression from the results obtained in the above-mentioned in vitro studies of imidacloprid is that cytotoxicity, cell viability and level of DNA damage remain largely unaffected at concentrations below 2 mM when the cells are of human origin.

It is difficult to compare the results between the present study and the study of Senyildiz et al. (2018). The latter authors used much higher concentrations of imidacloprid, and also much longer exposure times (24 or 48 h). However, like the U-937 lymphoma cells used in the present study, the HepG2-cells seemed rather resistant towards the cytotoxic and DNA-damaging effects of imidacloprid. The exposure time of 3 h used in the present study was selected based on the exposure protocols used in our previous studies on long-term cultures of human lymphocytes (Andersson et al.

**Table 3** Examples of different statistical approaches used when evaluating Comet data at our lab throughout the years

Indicator of DNA damage	Data set (pooled data)	“Pretreatment” of data	Data presented as	Statistical approach	References
Tail moment	Cells	None	Means	Kolmogorov–Smirnov two-sample test	Bergqvist et al. (1998)
Tail moment	Slides	None	Average medians	Two-tailed <i>t</i> test	Andersson et al. (2003)
Tail intensity	Slides	Extreme values excluded	Average medians	Two-tailed <i>t</i> test	Andersson et al. (2007)
Tail intensity	Cells	Log-transformation	Means	GLM + post hoc	Demma et al. (2009)
Tail intensity	Cells	None	Means	Mixed-effects ANOVA using maximum likelihood + post hoc	Durling et al. (2009)
Tail intensity	Cultures	None	Average medians	Two-tailed <i>t</i> test	Boldbaatar et al. (2014)

GLM generalized linear model

2003). Three hours is a common exposure time in gene mutation assays in vitro, such as the L51784 mouse lymphoma assay (TK-locus mutation assay), and is therefore probably relevant also in a comet assay evaluating primary DNA damage.

The potential genotoxicity of imidacloprid has also been investigated in vivo, and in these studies increased DNA damage was reported in rat sperm (Bal et al. 2012), in livers from tree frogs (Perez-Iglesias et al. 2014) and in zebra fishes (Ge et al. 2015). The only in vivo study on the potential genotoxicity of nitenpyram that was found in our literature survey was a study reporting an increased level of DNA damage in livers from zebra fishes (Yan et al. 2015).

Two different positive controls were used in the present study: chlorambucil when the cells were exposed without metabolic activation, and cyclophosphamide when S9 was added during the exposure. These compounds were partly chosen on the basis of the origin of the cell line U-937, and also because of their structural similarities. Both of them belong to the nitrogen mustard family of cytostatic agents and are approved for the treatments of several cancers of lymphocytic origin. Their main mechanism of action is alkylation of DNA and inter- and intra-strand crosslinking of the DNA strands. It was therefore believed that the U-937 cells would be highly susceptible to the treatment with these two cytostatic agents. However, since the findings in a series of pilot studies (data not shown) showed that the U-937 cells were rather resistant towards the cytotoxic and DNA-damaging effects of the direct acting chlorambucil, a rather high concentration (3 mM) was deliberately used for this positive control. The concentration of the indirect acting cyclophosphamide (100  $\mu$ M) was chosen based on our previous findings in a study on extended-term cultures of human lymphocytes (Andersson et al. 2003). Although U-937 cells have been used for mutagenicity testing of, for example, nanoparticles (Mannerstrom et al. 2016), they may not be ideal when monitoring potential DNA damage induced by neonicotinoids. The resistance of these cells towards the cytotoxic and genotoxic effects of our positive controls could partially

be explained by the fact that the cells are of a malignant origin and therefore genetic instable, lacking a fully functional p53 gene. Consequently, it can be expected that these cells contain and accumulate high frequencies of unknown genetic mutations (Sugimoto et al. 1992).

Usually the experimental design for a comet assay performed in vitro is a nested hierarchical design where the cells (comets) are nested within slides/gels, which themselves are nested within treatments which are nested within cultures/samples. Moreover, the distribution of comet data is usually highly skewed and often includes numerous comets without tails (which means that the data sets usually contain many zero values). Also, a typical set of comet data does not follow standard parametric distributions even after the data have been log-transformed (Verde and Rottmann 2015). All this makes the statistical evaluation of comet data quite challenging. Over the years there has been much work done to address these issues and several strategies for how to statistically analyse comet data have been suggested (Bright et al. 2011; Heberger et al. 2014; Lovell and Omori 2008; Moller and Loft 2014; Wiklund and Agurell 2003). For example, in 2011 Bright et al. published a set of recommendations of how to analyse comet assay data and they recommended that the best way to handle the skewness of the comet data was to log-transform and then aggregate the data on culture level before analysing the data set using one-way ANOVA. However, this suggestion assumes that the distribution after data transformation follow parametric distribution which is not always the case (Lovell et al. 1999). Nor does the approach suggested by Bright et al. take the hierarchical nature of the data into account.

After having used many different ways to analyse the comet data (Andersson et al. 2003, 2007; Bergqvist et al. 1998; Boldbaatar et al. 2014; Demma et al. 2009; Durling et al. 2009) generated in different studies (see Table 3), we decided to use an approach based on a proportional odds model tailored to continuous outcomes. After consulting a statistician, a model was built and used on our data from the neonicotinoid experiments. For comparative purposes, the

data were also analysed using a traditional one-way ANOVA on pooled cell data. As far as we know, the proportional odds model tailored to continuous outcomes which we have called Uppsala Comet Data Analysis Strategy (UCDAS) is a completely new approach to analyse comet data. UCDAS should be insensitive to a large number of zero values, it does not require log-transformation of the data, and the proposed model also addresses the hierarchical structure of data eliminating the need of data aggregation. Based on the results obtained in the present study, UCDAS seemed a little bit more conservative than the ANOVA, but overall, the two different strategies for analysing comet data basically gave the same results.

To conclude, based on the findings in the present study, the two neonicotinoids imidacloprid and nitenpyram seem to be of little concern when it comes to their potential DNA-damaging effects, at least in U-937 cells. However, since these cells were found to be rather resistant to the DNA-damaging effects of our two positive controls chlorambucil and cyclophosphamide, U-937 cells may not be the best cells to use when evaluating the potential genotoxicity of xenobiotics.

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## Compliance with Ethical Standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical Approval** This paper does not contain animal or clinical studies and patient data.

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## References

- Al-Sarar AS, Abobakr Y, Bayoumi AE, Hussein HI (2015) Cytotoxic and genotoxic effects of abamectin, chlorfenapyr, and imidacloprid on CHOK1 cells. *Environ Sci Pollut Res Int* 22(21):17041–17052. <https://doi.org/10.1007/s11356-015-4927-3>
- Andersson M, Agurell E, Vaghef H, Bolcsfoldi G, Hellman B (2003) Extended-term cultures of human T-lymphocytes and the comet assay: a useful combination when testing for genotoxicity in vitro? *Mutat Res* 540(1):43–55
- Andersson MA, Petersson Grawe KV, Karlsson OM, Abramsson-Zetterberg LA, Hellman BE (2007) Evaluation of the potential genotoxicity of chromium picolinate in mammalian cells in vivo and in vitro. *Food Chem Toxicol* 45(7):1097–1106. <https://doi.org/10.1016/j.fct.2006.11.008>
- Anuszczywska EL, Kozirowska JH (1995) Role of pyridine N-oxide in the cytotoxicity and genotoxicity of chloropyridines. *Toxicol In Vitro* 9(2):91–94
- Bal R, Naziroglu M, Turk G et al (2012) Insecticide imidacloprid induces morphological and DNA damage through oxidative toxicity on the reproductive organs of developing male rats. *Cell Biochem Funct* 30(6):492–499. <https://doi.org/10.1002/cbf.2826>
- Bergqvist M, Brattstrom D, Stalberg M, Vaghef H, Brodin O, Hellman B (1998) Evaluation of radiation-induced DNA damage and DNA repair in human lung cancer cell lines with different radiosensitivity using alkaline and neutral single cell gel electrophoresis. *Cancer Lett* 133(1):9–18
- Bianchi J, Cabral-de-Mello DC, Marin-Morales MA (2015) Toxicogenetic effects of low concentrations of the pesticides imidacloprid and sulfentrazone individually and in combination in in vitro tests with HepG2 cells and *Salmonella typhimurium*. *Ecotoxicol Environ Saf* 120:174–183. <https://doi.org/10.1016/j.ecoenv.2015.05.040>
- Boldbaatar D, El-Seedi HR, Findakly M et al (2014) Antigenotoxic and antioxidant effects of the Mongolian medicinal plant *Leptopyrum fumarioides* (L): an in vitro study. *J Ethnopharmacol* 155(1):599–606. <https://doi.org/10.1016/j.jep.2014.06.005>
- Bright J, Aylott M, Bate S et al (2011) Recommendations on the statistical analysis of the Comet assay. *Pharm Stat* 10(6):485–493. <https://doi.org/10.1002/pst.530>
- Calderon-Segura ME, Gomez-Arroyo S, Villalobos-Pietrini R et al (2012) Evaluation of genotoxic and cytotoxic effects in human peripheral blood lymphocytes exposed in vitro to neonicotinoid insecticides. *J Toxicol* 2012:612647. <https://doi.org/10.1155/2012/612647>
- Chlopkiwicz B, Wojtowicz M, Marczewska J, Prokopczyk D, Kozirowska J (1993) Contribution of N-oxidation and OH radicals to mutagenesis of 2-chloropyridine in *Salmonella typhimurium*. *Acta Biochim Pol* 40(1):57–59
- Cimino AM, Boyles AL, Thayer KA, Perry MJ (2017) Effects of neonicotinoid pesticide exposure on human health: a systematic review. *Environ Health Perspect* 125(2):155–162. <https://doi.org/10.1289/ehp515>
- Costa C, Silvari V, Melchini A et al (2009) Genotoxicity of imidacloprid in relation to metabolic activation and composition of the commercial product. *Mutat Res* 672(1):40–44. <https://doi.org/10.1016/j.mrgentox.2008.09.018>
- Demma J, Engidawork E, Hellman B (2009) Potential genotoxicity of plant extracts used in Ethiopian traditional medicine. *J Ethnopharmacol* 122(1):136–142. <https://doi.org/10.1016/j.jep.2008.12.013>
- Durling LJ, Busk L, Hellman BE (2009) Evaluation of the DNA damaging effect of the heat-induced food toxicant 5-hydroxymethylfurfural (HMF) in various cell lines with different activities of sulfotransferases. *Food Chem Toxicol* 47(4):880–884. <https://doi.org/10.1016/j.fct.2009.01.022>
- Feng S, Kong Z, Wang X, Peng P, Zeng EY (2005) Assessing the genotoxicity of imidacloprid and RH-5849 in human peripheral blood lymphocytes in vitro with comet assay and cytogenetic tests. *Ecotoxicol Environ Saf* 61(2):239–246. <https://doi.org/10.1016/j.ecoenv.2004.10.005>
- Ge J, Chow DN, Fessler JL, Weingeist DM, Wood DK, Engelward BP (2015) Micropatterned comet assay enables high throughput and sensitive DNA damage quantification. *Mutagenesis* 30(1):11–19. <https://doi.org/10.1093/mutage/geu063>
- Heberger K, Kolarevic S, Kracun-Kolarevic M et al (2014) Evaluation of single-cell gel electrophoresis data: combination of variance analysis with sum of ranking differences. *Mutat Res, Genet Toxicol Environ Mutagen* 771:15–22. <https://doi.org/10.1016/j.mrgentox.2014.04.028>

- Iwasa T, Motoyama N, Ambrose JT, Roe RM (2004) Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. *Crop Prot* 23(5):371–378. <https://doi.org/10.1016/j.cropro.2003.08.018>
- Jeschke P, Nauen R, Schindler M, Elbert A (2011) Overview of the status and global strategy for neonicotinoids. *J Agric Food Chem* 59(7):2897–2908. <https://doi.org/10.1021/jf101303g>
- Lovell DP, Omori T (2008) Statistical issues in the use of the comet assay. *Mutagenesis* 23(3):171–182. <https://doi.org/10.1093/mutag/e/gen015>
- Lovell DP, Thomas G, Dubow R (1999) Issues related to the experimental design and subsequent statistical analysis of in vivo and in vitro comet studies. *Teratog Carcinog Mutagen* 19(2):109–119
- Mannerstrom M, Zou J, Toimela T, Pyykko I, Heinonen T (2016) The applicability of conventional cytotoxicity assays to predict safety/toxicity of mesoporous silica nanoparticles, silver and gold nanoparticles and multi-walled carbon nanotubes. *Toxicol In Vitro* 37:113–120. <https://doi.org/10.1016/j.tiv.2016.09.012>
- Moller P, Loft S (2014) Statistical analysis of comet assay results. *Front Genet* 5:292. <https://doi.org/10.3389/fgene.2014.00292>
- Perez-Iglesias JM, Ruiz de Arcaute C, Nikoloff N et al (2014) The genotoxic effects of the imidacloprid-based insecticide formulation Glacoxan Imida on Montevideo tree frog *Hypsiboas pulchellus* tadpoles (Anura, Hylidae). *Ecotoxicol Environ Saf* 104:120–126. <https://doi.org/10.1016/j.ecoenv.2014.03.002>
- Sasaki YF, Nakamura T, Kawaguchi S (2007) What is better experimental design for comet assay to detect chemical genotoxicity? *AATEX J* 14:499–504
- Schenker R, Tinembart O, Humbert-Droz E, Cavaliero T, Yerly B (2003) Comparative speed of kill between nitenpyram, fipronil, imidacloprid, selamectin and cythioate against adult *Ctenocephalides felis* (Bouche) on cats and dogs. *Vet Parasitol* 112(3):249–254
- Senyildiz M, Kilinc A, Ozden S (2018) Investigation of the genotoxic and cytotoxic effects of widely used neonicotinoid insecticides in HepG2 and SH-SY5Y cells. *Toxicol Ind Health* 34(6):375–383. <https://doi.org/10.1177/0748233718762609>
- Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 175(1):184–191
- Su F, Zhang S, Li H, Guo H (2007) In vitro acute cytotoxicity of neonicotinoid insecticide imidacloprid to gill cell line of flounder *Paralichthys olivaceus*. *Chin J Oceanol Limnol* 25(2):209–214. <https://doi.org/10.1007/s00343-007-0209-3>
- Sugimoto K, Toyoshima H, Sakai R et al (1992) Frequent mutations in the p53 gene in human myeloid leukemia cell lines. *Blood* 79(9):2378–2383
- Sundstrom C, Nilsson K (1976) Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int J Cancer* 17(5):565–577
- Tice RR, Agurell E, Anderson D et al (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ Mol Mutagen* 35(3):206–221
- Tomizawa M, Casida JE (2005) Neonicotinoid insecticide toxicology: mechanisms of selective action. *Annu Rev Pharmacol Toxicol* 45:247–268. <https://doi.org/10.1146/annurev.pharmtox.45.1.2040>
- Union E (2018) Official Journal of the European Union, L 132, 30 May 2018 Official Journal
- Vaghef H, Wisen AC, Hellman B (1996) Demonstration of benzo(a) pyrene-induced DNA damage in mice by alkaline single cell gel electrophoresis: evidence for strand breaks in liver but not in lymphocytes and bone marrow. *Pharmacol Toxicol* 78(1):37–43
- Verde P, Rottmann A (2015) Statistical models to analyze genotoxicological experiments with the comet assay data. *Ann Biom Biostat* 2(3):1025
- Wiklund SJ, Agurell E (2003) Aspects of design and statistical analysis in the Comet assay. *Mutagenesis* 18(2):167–175
- Yan S, Wang J, Zhu L, Chen A, Wang J (2015) Toxic effects of nitenpyram on antioxidant enzyme system and DNA in zebrafish (*Danio rerio*) livers. *Ecotoxicol Environ Saf* 122:54–60. <https://doi.org/10.1016/j.ecoenv.2015.06.030>
- Zeljezic D, Mladinic M, Zunec S et al (2016) Cytotoxic, genotoxic and biochemical markers of insecticide toxicity evaluated in human peripheral blood lymphocytes and an HepG2 cell line. *Food Chem Toxicol* 96:90–106. <https://doi.org/10.1016/j.fct.2016.07.036>
- Zeljezic D, Vinkovic B, Kasuba V, Kopjar N, Milic M, Mladinic M (2017) The effect of insecticides chlorpyrifos, alpha-cypermethrin and imidacloprid on primary DNA damage, TP 53 and c-Myc structural integrity by comet-FISH assay. *Chemosphere* 182:332–338. <https://doi.org/10.1016/j.chemosphere.2017.05.010>

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