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Inhibition of lung surfactant function as an alternative method to predict lung toxicity following exposure to plant protection products

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1. Preface and acknowledgments

The present project “Inhibition of lung surfactant function as an alternative method to predict lung toxicity following exposure to plant protection products” studied how eleven plant protection products affected lung surfactant function, measured *in vitro*, and how they affected the breathing pattern of live exposed mice.

The project was financed by the Danish Environmental Protection Agency (EPA, Miljøstyrelsen) project number MST-667-00224, and was carried out in the period from July 2019 to September 2022 at the National research Centre for the working environment (NFA). The co-ordination and most of the work was done by senior researcher Jorid B. Sørli. Alexander C. Ø. Jensen measured aerosol size distribution and concentration in the *in vivo* experiments, Karin S. Hougaard followed the progress of the project and gave scientific input throughout the project period, and during the writing of the report and scientific paper describing the work.

Emilie Da Silva, Signe H. Nielsen, Eva Terrida, and Michael Guldbrandsen are thanked for excellent technical assistance in the lab.

Furthermore, the project would like to acknowledge the advisory board for “Sundhed og Pesticider” (“Health and pesticides”) for constructive discussions of the project, and in particular Louise Thorup Mundt and Anette Ravn Bharathan representing Danish Crop Protection Association and Mia Kjærstad and Louise Lundberg from the Danish EPA for their constructive comments and suggestions. Finally, we would like to acknowledge Henrik Frølich Brødsgaard at the Danish EPA for good collaboration during the project period.

2. Abstract

Currently acute inhalation toxicity is a required test for pesticide active ingredients (AIs) and formulated plant protection products (PPPs). As there are no accepted alternative methods for testing of this endpoint, animal experiments are required. The outcome of the test used for regulating the chemicals is “lethal concentration 50”, the concentration that will kill 50% of the exposed animals (LC_{50}). In this study, we investigated an alternative method for determining the effect of inhaled substances on the lungs. We studied 11 PPPs for their ability to inhibit lung surfactant (LS) function *in vitro*, and subsequently evaluated if this predicted changes in breathing patterns of exposed mice. Six of the eleven PPPs inhibited LS function, and eight changed the breathing pattern of exposed mice. Most of these caused changes indicative of sensory irritation (6), three caused changes indicative of pulmonary irritation and two caused a reduction in tidal volume (one product cause all three changes). In addition to the experimental work, we used two QSARs, the RespiraTox QSAR for respiratory irritation, and the Danish QSAR database for respiratory sensitisation in humans, to predict airway effects from the AIs and co-formulants in the PPPs. The RespiraTox QSAR predicted all the AIs that were suited for the QSAR (9/9) as irritants. When the AIs were tested in the Danish QSAR database for respiratory sensitisation in humans, one of the chemicals was predicted positive. Very little published information could be found on the effect of either the AIs or the co-formulants on the lungs. The results from the *in vitro* inhibition of LS function predicted changes in respiration of exposed mice with a sensitivity of 65% and a specificity of 66%. None of the products are classified and labelled as lung irritants (i.e. have either the label H334: May cause allergy or asthma symptoms or breathing difficulties if inhaled, or H335: may cause respiratory irritation) however two products were classified and labelled as “harmful if inhaled” (H332). These two products (A and D) both inhibited LS function *in vitro* and changed the breathing patterns of exposed mice. This project investigated if *in vitro* LS function inhibition correlated to changes in respiratory patterns in exposed mice. The overarching aim of the project group at NFA is to replace animal testing in acute inhalation toxicity testing for regulation. To this end, testing the effect on LS function could be included in a battery of *in vitro* tests predicting different toxicological endpoints related to effects in the respiratory system, but cannot stand alone as a replacement. This battery of tests has not been defined, however there is ongoing work from both regulatory bodies, industry and academia to define an integrated approach to testing and assessment (AITA) for testing effects on the respiratory system without using animal experiments. Testing the effect of inhaled substances on LS function is not a method accepted as an alternative to animal testing in regulatory guidelines, however it has been used to test a range of chemical classes, and these results have been compared to other *in vitro*, animal and human endpoints. These results have been published in peer-reviewed journals, and the test is part of an adverse outcome pathway AOP currently under evaluation by the OECD.

3. Dansk resume

Regulering af aktivstoffer i pesticider (AI'er) og formulerede plantebeskyttelsesmidler (PPP'er) kræver test for akut inhalationstoksicitet, som reglerne er i dag. Da der ikke findes accepterede alternative metoder (altså metoder som ikke anvender forsøgsdyr) udføres disse tests i dag på dyr, typisk rotter. Målet med dyreforsøgene er at finde LC_{50} (Lethal Concentration 50), den aerosolkoncentration som ved indånding tager livet af halvdelen af de eksponerede dyr. I dette projekt brugte vi en alternativ metode til at forsøge forstå hvordan kemiske stoffer påvirker lungerne. Vi undersøgte 11 PPP'er for om de hæmmede funktionen af lungesurfaktant (LS) *in vitro*. Efterfølgende evaluerede vi, om dette forudsagde ændringer i åndedrætsmønstre hos eksponerede mus. Resultaterne fra *in vitro* testen prædikterede ændringer i respirationen hos eksponerede mus med en sensitivitet på 65 % og en specificitet på 66 %. Ingen af produkterne er mærket som irriterende for luftvejene (dvs. har faresætningerne H334: Kan forårsage allergi- eller astmasymptomer eller åndedrætsbesvær ved indånding eller H335: Kan forårsage irritation af luftvejene). To produkter var dog klassificeret som "skadelige ved indånding" (H332). Disse to produkter (A og D) hæmmede begge LS-funktionen *in vitro* og ændrede vejrtrækningsmønstrene hos eksponerede mus. Vi brugte derudover to QSAR'er, "RespiraTox QSAR" for luftvejsirritation og den danske QSAR-database for "respiratorisk sensibilisering hos mennesker", til at modellere om AI'erne og hjælpestofferne i PPP'erne har effekter på luftvejene. RespiraTox QSAR prædikterede at alle AI'er har en irriterende effekt på luftvejerne. Den danske QSAR-database prædikterede et af AI'erne til at have en respiratorisk sensibiliserende effekt i mennesker. Ved litteratursøgning fandt vi meget lidt information om både AI'ers og hjælpestoffers virkning på lungerne. Hvis forsøg på dyr for at bestemme om der en akut effekt på lungerne i regulering skal erstattes, bliver man nødt til at forstå de forskellige skadelige effekter som indånding af kemikalier kan have på åndedrætssystemet og de underliggende mekanismer. Derefter kan man sammensætte et batteri af tests som sammen kan erstatte dyreforsøg. Et sådant batteri er dog endnu ikke blevet defineret, men arbejdet er i gang både fra et regulatorisk, akademisk og industrielt synspunkt. En af de tests som kan indgå i sådan et batteri er test af effekten på LS-funktionen, som også anvendt i dette projekt. At teste effekt på LS-funktionen er endnu ikke et accepteret alternativ til dyreforsøg i regulatoriske retningslinjer, men det er blevet brugt til at teste en række forskellige kemiske stoffer og blandinger, og disse resultater er blevet sammenlignet med resultater fra andre *in vitro* forsøg, og effekten på dyr og mennesker. Det hidtidige arbejde er publiceret i peer-reviewede tidsskrifter, og er samlet i en såkaldt adverse outcome pathway (AOP) som er under evaluering af OECD.

4. Background and hypothesis of the project

Currently the development and regulation of active ingredients (AIs) and formulated plant protection products (PPPs) depend on performing animal experiments to assess the safety for humans for some endpoints. Where alternatives to animal experiments are available, these are used, however for the endpoint of acute inhalation toxicity, there are no accepted alternatives. Replacing this animal test with a (yet to be defined) battery of *in vitro* test would aid in both product development, and regulation of AIs, PPPs and chemicals that are covered by other regulations.

We hypothesised that:

- Results from an *in vitro* method for inhibition of lung surfactant function (LS, an essential bio-fluid in the lungs) would correlate to *in vivo* changes in breathing patterns of exposed mice.
- That using LS inhibition testing could form a basis for eventual replacement of animal experiments both during product development and regulatory testing of AI and PPPs.

We addressed these hypotheses by:

- Testing eleven PPPs available on the Danish market for their inhibitory effect on LS function *in vitro*.
- Testing the same PPPs by exposing mice and analysing changes in breathing patterns.
- Comparing the results from the *in vitro* and *in vivo* experiments to test the accuracy of the prediction from the *in vitro* test.
- In addition, we used two publically available QSAR models for effects on the lungs to explore if there was a correlation between the predictions and the *in vitro* and *in vivo* results.

The experimental results in this report have been collected for publication in a scientific journal.

5. Introduction

Development of new active ingredients (AIs) and plant protection products (PPPs), and their subsequent regulation, currently depends upon testing on animals for specific endpoints. Despite extensive focus and research into alternatives to animal experiments, some areas of toxicity testing have proven difficult to replace. In this project, we have focused on acute inhalation toxicity as it is one of these problematic areas.

In the current regulatory context, the acute inhalation toxicity of AIs and PPPs should be reported, e.g. if the products are applied by spraying, according to the data requirement Regulations (EU) 283/2013 [1] and 284/2013 [2]. However, the PPP Regulation (EC) 1107/2009 also states, “The development of non-animal test methods should be promoted in order to produce safety data relevant to humans and to replace animal studies currently in use.” [3]. Assessment of acute inhalation toxicity currently depends upon toxicity testing in animals, as there are no validated and accepted non-animal tests available for regulatory use. This end-point can be evaluated by three OECD Test Guidelines (TG) for acute inhalation toxicity testing: TG403, TG436, and TG433 [4-6]. According to these TGs, groups of animals, typically rats, are subjected to a high aerosol concentration of the test substance for up to four hours. Thereafter the animals are returned to their cages and observed for two weeks. The end-point during these two weeks is the death of the animal for the two oldest and most frequently used guidelines (TG403 and TG436). A refinement of the guidelines (TG433) instead uses “evident signs of toxicity” as the endpoint, these include in the most predictive order: tremors, hypo-activity, bodyweight loss >10%, and irregular breathing [7]. Death, or evident sign of toxicity are used to set an LC50, the concentration that will kill 50% of the animals. The LC50 is used to classify the test compound into different categories of toxicity.

In the TGs the exposure is via the lungs, but the cause of death can be from toxicity either originating in the lungs, or systemic toxicity. Attempts have been made to estimate LC50 after inhalation e.g. based on already available data. In the CLP regulation[1] ((EC) 1272/2008) [8] the assessment of acute inhalation toxicity of mixtures can be calculated as an acute toxicity estimate (ATE). This method calculates the ATE by adding up the estimated toxicity of each of the known mixture ingredients according to their concentration. In addition, the European chemical agency (ECHA) has published guidance on the application of CLP criteria and this guideline includes conversion factors for extrapolation of acute toxicity data from other routes of exposure, e.g. from oral administration to inhalation [9]. Kurth et al [11] used the ATE method to assess PPPs for inhalation toxicity by adding the inhalation toxicity of the individual constituents in the products. The PPPs had already been tested in animals according to OECD TGs for authorization in Germany. When the ATE estimates were compared to the inhalation testes performed with the PPPs, the ATE calculation failed to predict the acute inhalation toxicity hazard in more than half of the cases. Most importantly, the ATE underestimated the hazard classified by the *in vivo* method in 45% of the cases [11]. This suggest that the toxicity of the individual ingredients are not additive, but can be synergistic when they occur in a mixture. This conclusion is supported by a similar study performed on PPPs tested for regulatory purposes in the US [10].

Furthermore, it has proven challenging to substitute animal-based tests with non-animal alternative methods. A major problem in this regard is that the OECD TGs are based on systemic

[1] The CLP regulation governs the classification, labelling and packaging of chemicals and mixtures for protection of human health and the environment

toxicity, whereas the majority of the *in vitro* methods aim to recreate parts of the exposed organ, the lungs. The lungs have a complicated structure, and the cell type, cell composition, and architecture differs throughout the organ. This presents a challenge with respect to which regions of the lungs that should be recreated *in vitro* [8]. When inhalation toxicity tests are performed about 90% of the tested compounds induced local effects on the lungs at lower concentrations than those causing 50% lethality [9]. This indicates that the more feasible strategy for replacing animal tests, namely predicting the effects on the lungs *in vitro*, would be useful. The choice of test system should preferably be guided by mechanistic understanding of how local effects can lead to adverse effects on the lungs that ultimately affect the whole organism. This can be done by describing how an initial event can lead to a cascade of effects ending in an adverse outcome, a so-called adverse outcome pathway (AOP).

Deposition in the lungs after inhalation is affect by several factors including the aerodynamic particles size and density, water solubility, reactivity and concentration of chemicals in the aerosol [11]. Next generation risk assessment, i.e. risk assessment without using animal experiments is exposure lead, hypothesis driven and human relevant [12]. Depending on the deposition pattern, the aerosol may interact or damage different areas and cell types of the lungs, activate the immune defences, or interact with the nerves innervating the lungs. If the aerosol created, e.g. by spraying a PPP, results in small enough particles (between 0.005 and 0.5 µm in aerodynamic diameter) these can reach and deposit in the deepest parts of the lungs, the alveoli [13]. The inner surfaces of the alveoli are coated by a thin layer of liquid containing lung surfactant (LS). LS is a mixture of phospholipids and lung surfactant associated proteins (SP-A, -B, -C and –D) secreted by type II alveolar cells [14, 15]. The LS covers the air-liquid interface, and lowers surface tension of the interface of the alveoli. With each in- and out-breath (compression and expansion of the lung surface area) the LS regulates surface tension, so that it is very low when the surface area is at its lowest, and the walls of the alveoli come close to each other (during exhalation). The regulation of surface tension makes breathing effortless, and LS is essential for normal lung function.

The *in vitro* test system allows exposing LS to the test substance outside the lungs. A drop of LS sits inside an exposure chamber, and the surface area of the drop is change to an extent and at a frequency that simulates breathing. By measuring the change in surface tension of the "breathing" drop, the effect of the substance exposure can be determined. LS regulates surface tension throughout the breathing cycle, however the minimum surface tension, the tension when the alveoli are at their smallest, is the most important parameter. In this study we exposed the LS to an aerosol of the PPP to determine how it affects the LS function, by determining the change in minimum surface tension. We have earlier shown that the effect of airborne substances on LS function *in vitro*, correlates strongly with the effects of the same substances on the breathing pattern and decreased lung function of exposed animals [16-19]. The predictiveness of LS function inhibition *in vitro* of effects on intact lungs (summarized in table 1) depend on several factors including the type of product or chemical and which lung effects are used for comparison.

TABLE 1. The predictivness of LS function measurement *in vitro* for the effect of the exposure in animals or humans.

Chemical/product	**n**	***In vitro* test**	***In vivo* results**	**Predictivness of *in vitro* results**
Impregnation product[1]	21	LS inhibition	Breathing pattern Human data¤	Sensitivity: 100% Specificity: 63% Accuracy: 86%
Inhaled pharmaceutical[2]	10	LS inhibition	Breathing pattern	Sensitivity: 100%

			Safe use Human data*	Specificity: 100% Accuracy: 100%
Single chemicals[3^]	26	LS inhibition	Change in respiration[§]	Sensitivity: 81% Specificity: 100% Accuracy: 81%

[1]: [17] [2]: [18, 19] [3]: [16], n: number of chemicals/products tested, breathing pattern changes measured in plethysmograph, safe use by inhaled pharmaceuticals on the market, human data ¤from people that have become intoxicated during use, or *measured in lung lining fluid during lung damage, ^single chemicals tested for GHS classification for acute inhalation toxicity, § change in respiration assessed cage side

The cascade of events that starts when a chemical or mixture of chemicals inhibits LS function and ends with decreased lung function has been collected in an AOP "LS function inhibition leading to decreased lung function" (Fig. 1, [20] and www.aopwiki.org/aops/302). When the LS is damaged, its function is inhibited and a high surface tension at minimum surface area can cause the alveoli to collapse. Reopening the alveoli, by forcing in air with inspiration, can damage the alveolar epithelial barrier further and leading to bleeding into the lungs (Fig. 1). Blood components further inhibit LS function. If, on the other hand, the alveoli remain collapsed, this reduces the area for gas exchange.

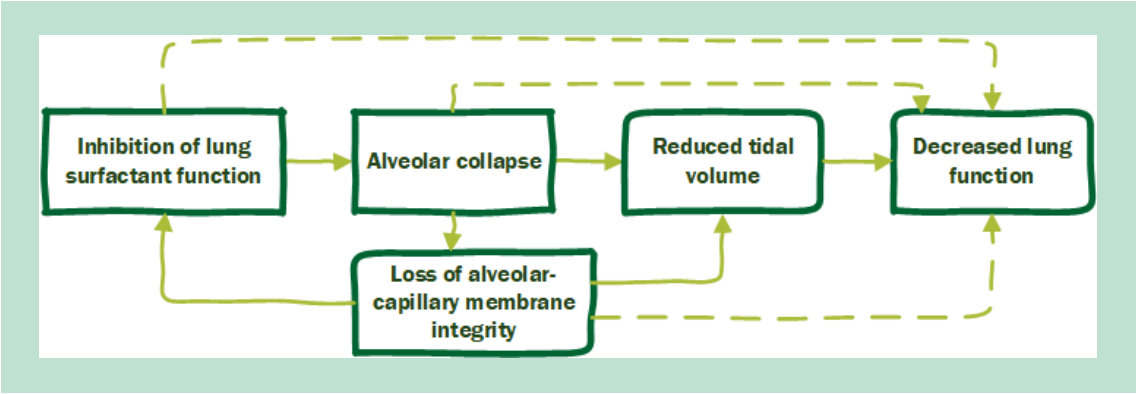

FIGURE 1. The adverse outcome pathway (AOP) starting with the inhibition of LS function that can lead to decreased lung function. If LS function is disrupted this can lead to collapse and subsequent reopening of the alveoli. The former leads to reduced tidal volume and the latter to loss of alveolar-capillary membrane integrity. The overall outcome of the cascade is decreased lung function [20].

To test our hypothesis, that "results from *in vitro* inhibition of LS function will correlate to *in vivo* changes in breathing patterns of exposed mice", we exposed mice to the same PPPs as tested *in vitro*. In the *in vivo* assay mice were placed in whole body plethysmographs, and exposed head out in the exposure chamber. Changes in respiration were monitored during the exposure, and several parameters can be determined from the breathing pattern (extensively described in [21]). These include the volume of each breath (tidal volume), breathing rate, how long it takes between breathing out until the next in-breath (time of pause) and between breathing in and the next out-breath (time of break). The changes in these parameters have been linked to different effects on the lungs, e.g. sensory irritation and pulmonary irritation [22]. Sensory irritation comes from the stimulation of the trigeminal nerve endings of the upper respiratory tract. In humans, it is described as a burning and painful sensation. Pulmonary irritation is caused by stimulation of nerve endings at the alveolar level [21]. Changes in breathing are also registered during OECD TG studies. However, assessment of these are made cage side, by observation of the animals, and these observations are much less specific and sensitive than those that can be obtained from plethysmography readings [16, 23]. It is currently not

known which breathing parameters are directly influenced by disruption LS function. The most logical change is reduced tidal volume (caused by collapsed alveoli), however the collapse and reopening of the alveoli can trigger nerve endings at the alveolar level. In this project, we have compared LS function inhibition to change in breathing rate, tidal volume, time of pause, and time of break.

Replacing animal experiments with *in vitro* experiments will still require experimental work. If the work can be done by computer models (i.e. Quantitative structure-activity relationship, QSAR) this would however further lower the burden of work associated with developing and regulating new PPPs. The ideal QSAR in this case would predict reduced lung function, however this QSAR does not exist to our knowledge. We identified two publically available QSAR models (RespiraTox developed by Fraunhofer ITEM and the Danish QSAR database) that predict adverse effects on the lungs. We used the models to predict the outcome of the active ingredients (AIs) and known co-formulants in the tested PPPs.

The first step in this project was to evaluate the Danish market for PPPs used. Selected PPPs were acquired (Fig. 2). The products were tested for their effect on LS function *in vitro*, and subsequently for how they affected the breathing of exposed mice. We performed a literature review of the AIs and co-formulants in the products to identify if effects on the lungs had been described previously, and assessed if the two QSARs predicted effects seen in this project (Fig. 2).

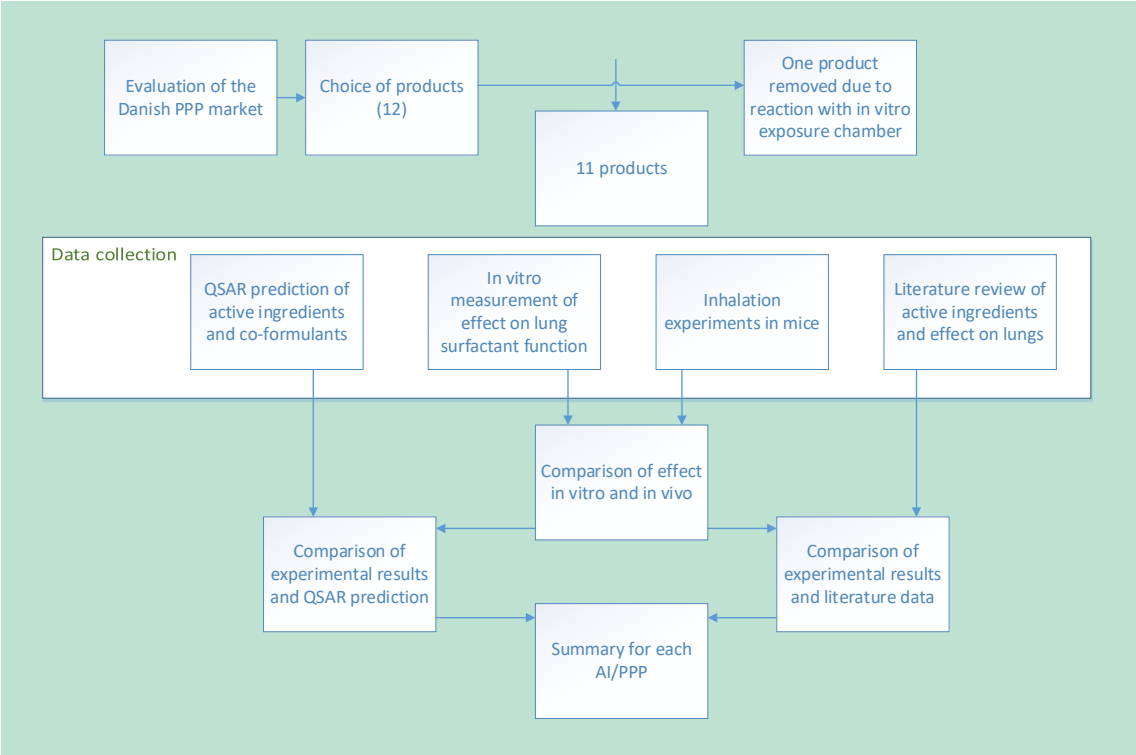

FIGURE 2. Overview of the project. The project started with an evaluation of the Danish market for PPPs, followed by choice of test products. Eleven products were run through two different lung related QSARs, tested for inhibition of LS function *in vitro* and effect on breathing patterns of exposed mice. A literature search was performed to obtain additional knowledge on effects on the lungs. Finally, the collected data was compared and summarized.

6. Materials and methods

6.1 Choice and composition of PPPs for testing *in vitro* and *in vivo*

When the grant application was written, we made a preliminary selection of AIs to test in the project based on statistics from the Danish EPA on use of PPPs in Denmark in 2015. We combined the most used AIs and the AIs with the highest environmental impact (defined by the Danish EPA as "Pesticidbelastningsindikator", described here: https://mst.dk/kemi/pesticider/anvendelse-af-pesticider/forbrug-af-pesticider-statistik-og-indikatorer/pesticidindikatorer/). We identified products containing these AIs from The Danish Authorised Pesticides database (https://mst.dk/kemi/database-for-bekaempelsesmidler/bmd/). However, when the project was initiated the original list had to be changed for two reasons: 1) the use of PPPs had changed according to the data available up until 2015, and 2) the sale of PPPs for professional use is under strict regulation and we bought/were donated what was available for purchase at the initiation of the project. Thus, we acquired 12 products spanning the categories of herbicides (3), fungicides (8) and insecticides (1). The products contained at total of 11 different AIs, most had one AI, two products contained two AIs. One product, the only insecticide, was incompatible with the *in vitro* test, and did therefore not proceed to testing. In this project we therefore tested 11 products in the categories of herbicides and fungicides, with a total of 10 different AIs in different formulations. Each was assigned a letter, the AI, use, formulation and respiratory health hazard statement is summarised in table 2.

TABLE 2. The name, CAS number and percentage of the AI in each product is summarized together with the use, formulation and hazard statement related to inhalation.

Product	AI	CAS	AI, %	Use	Formulation	CLP classification of PPP related to inhalation
A	azoxystrobin	131860-33-8	25%	Fungicide	Liquid	H332: harmful If inhaled
B	boscalid	188425-85-6	23%	Fungicide	Suspension concentrate	
	epoxiconazol	133855-98-8	7%			
C	boscalid	188425-85-6	50%	Fungicide	Water dispersable granulat	
D	Pyraclostrobin	175013-18-0	20%	Fungicide	Emulsion concentrate	H332: harmful If inhaled
E	Cycloxydim	99434-58-9	10%	Herbicide	Emulsion concentrate	
F	Glyphosate	1071-83-6	49%	Herbicide	Water soluble concentrate	
G	Metconazole	125116-23-6	9%	Fungicide	Emulsifiable concentrate	
H	Kresoxim methyl	143390-89-0	50%	Fungicide	Water dispersable granulat	

I	Propyzamid	23950-58-5	44%	Herbicide	Suspension concentrate
J	Pyrimethanil	53112-28-0	40%	Fungicide	Suspension concentrate
K	boscalid	188425-85-6	27%	Fungicide	Water soluble granulate
	Pyra-clostrobin	175013-18-0	7%		

In addition to the AI, formulated PPPs contain ingredients (so called co-formulants) to help the spreading of and increase efficacy of the product, e.g. wetting, anti-foaming, and dispersing agents, preservatives, emulsifiers and anti-oxidants. In table 3 the concentrations of different co-formulants have been collected from the product MSDS. The products were tested to determine if they could be aerosolized as the neat product before *in vitro* and *in vivo* tests, to determine the highest concentration that could be aerosolized. As several products were formulated as granulates, these were dissolved in water before testing to determine the highest concentration that could be aerosolised. The products were tested either undiluted or diluted in water, the dilution was the same *in vitro* and *in vivo*, except for products C, F and K (supplementary table 2). The aerosolization test aimed as a maximum aerosol concentration, and therefore the concentrations are significantly higher than the maximal approved doses on the labels of the PPPs.

TABLE 3. The chemical composition of each product divided by functional category of co-formulants (chemicals in each group can be found in supplement). Amount in % of product, data collected from MSDS.

	Wetting agent	Emulsifier	Solvent	Preserva-tive	Anti-freeze	Thickener	Not listed in MSDS
A	23				12		40
B		25		0.05	10	5	30
C						20	30
D	20	4	56				
E		5	61				24
F*							51
G	60				40		
H						30	20
I					5		51
J		10		0.05	5		45
K		5				30	35

*F did not have any co-formulants mentioned in MSDS

6.2 LS function measurements and determination of inhibitory dose

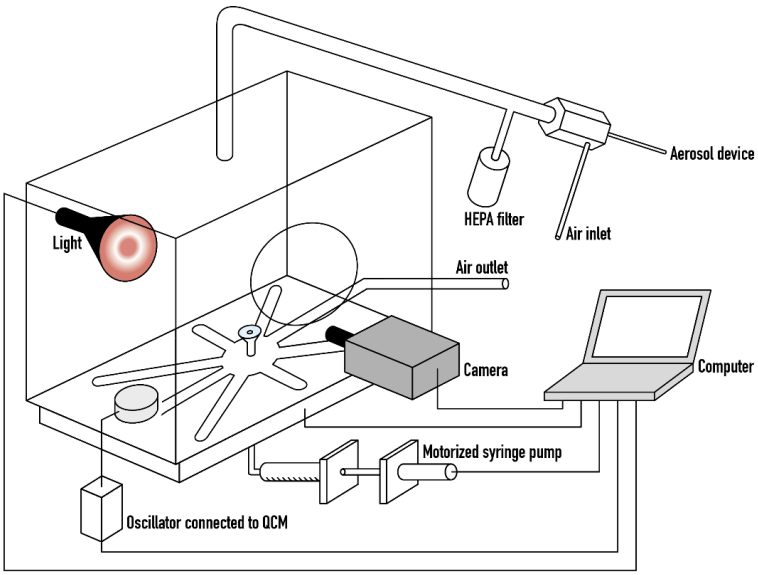

FIGURE 3. A graphical overview of the constrained drop surfactometer, adapted from [19]

LS function was measured using the constrained drop surfactometer. Shortly, LS has to be cycled at a frequency and change of area similar to breathing intact lungs to observe the surface tension lowering effects. This is done by computer controlled motion of a motorized syringe pump that adds and removes liquid of the LS droplet (Fig. 3). After a baseline period, the cycling drop is exposed to the test substance (Fig. 3), and the surface tension of the drop is calculated by drop shape imaging [24]. Inhibition of LS function is here defined as an increase in the minimum surface tension upon compression reaching values above 10 mN/m. The experiments were carried out as follows: A droplet of LS (10 µL of 2.5 mg/ml Curosurf, Chiesi, Parma, Italy), in a buffer containing 0.9% NaCl, 1.5 mM CaCl2, and 2.5 mM HEPES, adjusted to pH 7.0, was placed on a hollow pedestal. Curosurf is made from solvent extracted minced porcine lung tissue and contains ~99% w/w phospholipids and 1% w/w hydrophobic surfactant-associated proteins (SP-B and SP-C) [25]. The pedestal was con-nected to a motorized syringe pump that adds and removes liquid from the droplet at a de-fined volume and frequency. The droplet was cycled with a change of surface area of 25.9± 4.4% and at 3-second cycles to simulate breathing lungs. During the experiment, a camera took ten pictures per second of the backlit drop. The ADSA (axisymmetric drop shape analy-sis) software [24] was used to analyse the pictures to calculate the surface tension of the droplet. The pressurized air in the nebulizer and the exposure chamber were heated, and the temperature inside the exposure chamber was monitored using the TinyTag Plus 2 data logger (TGP-4017, Gemini Data Loggers Ltd, United Kingdom). The mean temperature in the experiments was 33.9±1.5 °C, on separate experiment days the temperature was stable within one degree C. A quartz crystal microbalance (QCM, Vitrocell, Waldkirch, Germany) was positioned close to the cycling LS

droplet. The QCM measurements were used to esti-mate the dose of each product that inhibited LS function. LS function was defined as being inhibited if at least three consecutive minima in surface tension were larger than 10 mN/m. The time of inhibition i.e., the first minimum above 10 mN/m, was combined with data from the QCM to estimate the inhibitory dose. At the time of inhibition the deposited mass on the QCM was recorded and converted to the mass deposited on the LS droplet by multiplying with the average surface area of the drop throughout the experiments (0.18 cm2).

Example of calculation of inhibitory dose: LS function was inhibited when 920 ng was de-posited on the QCM, to find the deposited mass on the LS drop this was multiplied with the surface area of the drop (0.18 cm2), here resulting in 166 ng/cm2. To estimate the substance amount per mass of LS this was divided by 0.025 mg (the droplet is 10 µL of 2.5 mg/ml LS) here resulting in 6626 ng/mg LS, or 6.63 µg/mg LS.

6.3 Animals

Mice were exposed to the same PPPs tested *in vitro* for lung surfactant function inhibition. A total of 191 inbred BALB/cJ male mice, aged 6–7 weeks at arrival, were purchased from Janvier (France) and housed in clear 1290D Euro standard type 3 polypropylene cages (380x220x150 mm) furnished with aspen bedding, enriched with small aspen blocks (Tapvei, Estonia) and nesting material (Enviro Dri, Lillico, Biotechnology, UK Tapvei). The photoperiod was from 06:00 to 18:00, and the temperature was 21°C and relative humidity in the animal room was 55%. Cages were sanitized twice weekly. Food (Altromin no. 1324, Altromin, Lage, Germany) and municipal tap water were available ad libitum. The mice were randomly assigned to cages at arrival, 3–4 mice per cage, and acclimatized for a minimum of one week prior to experiments. The exposures were done between 08:00 and 13:00. The animals were killed by cervical dislocation immediately after the end of exposure.

6.4 Ethical statement

Treatment of the animals followed procedures approved by The Animal Experiment Inspectorate, Denmark (Permissions No. 2019-15-0201-00114). All experiments were performed by trained personnel and conformed to the Danish Regulations on Animal Experiments (LBK nr. 474 af 15/05/2014 and BEK nr 12 af 07/01/2016), which include guidelines for care and use of animals in research. Anaesthesia was not used during the experiments, because measuring respiration depends on the animals being fully awake with uncompromised breathing. The exposure was stopped if the tidal volume (VT) was reduced by >50% compared to baseline during the exposure or after a maximum of 1.5 h of exposure.

6.5 *In vivo* exposure experiments

All animal experiments followed the same general setup as follows. Before placing the mice in the whole body plethysmographs, the aerosol generator was started to generate the level of noise that the mice would be exposed to throughout the experiment. Then the mice were weighted and placed in plethysmographs of the corresponding size. The plethysmographs were placed with the mice head out in a 20L stainless steel exposure chamber and their breathing monitored throughout the experiment in real-time using the Notocord-hem data acquisition software (Notocord Systems SA, Croissy-sur-Seine, France). The mice were given clean air to breath for at least 15 minutes prior to starting exposure, this was designated the baseline measure, and allows changes in breathing to be compared to parameters during non-exposure. After the baseline period, the mice were exposed to an aerosol of the test substances (either in increasing concentrations or at the same concentration, see below for details). After a maximum of 90 minutes of exposure, if the breathing had changed noticeably during the exposure, the mice were given 15-30 min of fresh air to breathe to see if the change was reversible. If there were no noticeable changes due to exposure, the mice were removed

immediately after exposure. After end of the experiment, the mice were killed by cervical dislocation, and the data analysed for changes in breathing parameters. Changes in the following parameters were analysed; tidal volume (VT, mL) i.e. how much air is drawn into the lungs with each breath, breathing rate (breaths per minute, BMP), the break between inhalation and exhalation (TB, ms), the pause between exhalation and the next inhalation (TP, ms). A comprehensive description of breathing pattern analysis and interpretation has been reported elsewhere [26-28].

The experiments were done in two steps, following two distinct experimental setups described below, to determine the lowest concentration (lowest observed adverse effect concentration, LOAEC) in the air that caused a change in breathing pattern compared to baseline parameters.

Firstly, we performed **concentration-range experiments** for each PPP. The concentration-range experiments aimed at finding the concentration level where effects were seen on breathing pattern. In these experiments mice (n=2-6) were exposed to the PPP, starting at the lowest infusion rate where we had observed effect with previously tested chemicals. This was done to start the exposure at a concentration level where we did not expect any toxic effect. After 10 min of exposure, the infusion rate was tripled, and this was repeated until 1) a clear effect on tidal volume was seen (only tidal volume was monitored in real-time), 2) until the aerosol generator could not aerosolize the liquid, or 3) after 90 min of exposure. If the mice did not have a clear reduction in tidal volume after 90 min of exposure, and the aerosol device had not reached its limit, a new concentration-range experiment was performed as described, starting with the highest infusion rate tested in the previous experiment. We defined adverse effects of PPPs as follows: 1) a ≥30% change compared to baseline in tidal volume or breathing rate lasting more than 5 min as an adverse effect, or 2) a ≥30% increase compared to baseline in TB or TP lasting more than 5 min as an irritating effect, 3) a combination of 1 and 2 as an adverse and irritating effect (see discussion for considerations of this definition).

Secondly, the concentration-range experiments were followed by **LOAEC experiments** to determine a LOAEC level. Groups of mice (n=4-7) were placed in the exposure chamber and after baseline measurement, the mice were exposed to the lowest concentration resulting in a change of breathing pattern from the concentration-range experiments. The mice were exposed for 1h, or for as long as the aerosol device could aerosolize the infused liquid (15-60 min). If there was a reduction in tidal volume >50% during this period, exposure was terminated.

6.6 Aerosol generation and measure

For air exposure to PPP, both *in vitro* and *in vivo*, aerosols were generated by filling a syringe with the test liquid and then passing the liquid into a Pitt no. 1 jet nebulizer [29] where the product was aerosolized with pressurized air. The product was led from the glass syringe by an infusion pump (Legato 100, Buch & Holm A/S, Denmark). In the *in vitro* assay the aerosol was led from the nebulizer through glass tubing into the 1.9L exposure chamber. The bottom of the chamber has holes where hollow channels suck air out of the chamber, and through a HEPA filter, before release into the atmosphere. During the *in vivo* inhalation exposure, the aerosol was lead from the nebulizer via glass tubing into a 20 L mouse exposure chamber [31]. Outlet air was passed through a series of particle- and active coal-filters be-fore the exhaust to the atmosphere.

Aerosol particle number concentrations and aerodynamic particle size distributions were measured using an Electrical Low Pressure Impactor (ELPI, HR-ELPI+, Dekati, Finland). The ELPI measures particles in the size range between 6 nm and 10 µm in 14 channels with one second time resolution. In the *in vitro* chamber, aerosols were measured to determine size resolution, but the deposited dose was assessed by QCM. In the inhalation experiments, the ELPI

measurements were used to estimate exposure concentrations. The measurements in the mouse exposure chamber were done at the same infusion rates as the mice were ex-posed to, however the experiments were done without animals in the chamber, as the aero-sol measurements would have disturbed the exposure of the animals. At high infusion rates the aerosol sampled from the chamber was diluted 12.5 times using a VKL10 diluter (VKL10, Palas GmbH, Karlsruhem, Germany) before detection by the ELPI.

During animal exposure, the PPP mass concentrations in the exposure chamber were meaured by gravimetric filter sampling during the LOAEC experiments according to stand-ard DS/EN 481 [30]. Aerosols were collected on pre-weighed Teflon filters (Flouropore™ Membrane filters, pore size 0.45 µm, Millipore A/S, Denmark) at a flow rate of 2 L/min using an Apex2™ personal sampling pump (Casella, Buffalo, USA).

To estimate the LOAEC in deposited mass in the alveoli of the exposed mice, we used the mass deposited on the filter during the LOAEC experiment to calculate the aerosol concentration in mg/m3. As the breathing rate and tidal volume was measured during the exposure we used these to estimate how much of the PPP the mice inhaled during the exposure. As the particles formed during aerosol formation were almost all respirable (supplementary figure 1) we assumed that the mice inhaled the aerosol concentration measured by the filter sample. We assumed that 10% of the inhaled aerosols were deposited in the alveolar region of the lungs, and that there was no clearance during the exposure time (15-60 min). We also assumed that a mouse has a total of 0.15 mg LS (for details of calculation see [18]). The exact number of animals used to test the toxicity of each PPP product can be found in Supplementary Table 2. The numbers of mice used for each PPP ranged from 12-31, the variation is due to 1) when toxicity occurred, 2) how variable the response was, 3) if experiments had to be rerun.

6.7 Collection of background knowledge of the PPPs

6.7.1 Literature search

To retrieve literature on the effect of inhaling AIs or co-formulants the following terms were used in PubMeb searches; #chemical name or CAS number AND #inhalation, and # chemical name or CAS number AND #lung. The EU registration and EFSA evaluations of all the AIs were collected and data for respiratory effects (LC_{50}, and/or description of experiments forming the basis for the evaluation or CLP classification) was noted. For the co-formulants the CAS number was used to find the submitted dossier in REACH (if any) and data used for classification on lung effects were collected.

6.7.2 ICE database

The ICE database collects data on acute toxicity studies submitted to U.S. EPA for PPP registration. The database was filtered for “Acute Inhalation Toxicity Assay (*in vivo*)”. The data includes the amount of active ingredient in the product, but none of the co-formulants. It contains LC_{50} data, and in some cases data on signs of toxicity. The database was used to search for any respiratory effects of PPPs containing the AI used in this project. This search was included in the original application to the Danish EPA, however using the database we realised that the information was limited to only active ingredients, and the retrieved information was not informative in this project. None the less, as the searches have been performed we have included the information in supplementary table 2.

6.7.3 QSARs for effects on the respiratory system

We identified two publically available QSARs for effects on the respiratory system: ResiraTox and the Danish QSAR database model for respiratory sensitization.

The RespiraTox model was developed to predict respiratory irritancy of single chemicals and mixtures [31]. The model has high sensitivity, i.e. it predicts irritants as irritants with high probability (96.1%), however, the model has very low specificity, i.e. it has a high rate of false positives, predicting non-irritants as irritants (23.6%). This is described as a worst-case scenario, however it results in high over-prediction of irritants [31]. RespiraTox focuses on airway irritation, an effect that likely occurs shortly after inhalation, and can be divided into sensory irritation (affecting the upper respiratory tract) and pulmonary irritation [21]. The QSAR is based on about 2000 chemicals [31].

The Danish QSAR database model for respiratory sensitization predicts effects that happen shortly after exposure (a decrease in forced expiratory flow in the first second, FEV1[2] ≥20% within 24h of exposure), but that requires previous exposure [32]. The model is based on 80 substances in the training set and has a sensitivity of 68.2-91.7 (depending on the model software) and a specificity of 88.8-93.9 (https://qsardb.food.dtu.dk/db/index.html).

Input for the QSARs was SMILEs (RespiraTox) or CAS numbers (Danish QSAR database) for AIs and co-formulants. The output from RespiraTox was “irritant” or not, i.e. if the substance would be irritating to the airways if inhaled. The Danish QSAR database output is; predicted to be a sensitizer (POS), not predicted to be a sensitizer (NEG), inconclusive (INC), these in combination with if the chemical is in or out of the domain of the QSAR (_IN or _OUT).

[2] FEV1 is the amount of air that can be exhaled during the first second of a forced breath after a maximum inhalation. The measure can be used to categorize the severity of obstructive lung disease such as asthma.

7. Results

The effect of the tested PPPs on LS function and on breathing patterns of exposed mice are summarized in table 4. This is followed by the predictions made for AIs and co-formulants by two QSAR models (table 5). Data for each product is collected in table 6.

When the PPPs were tested *in vivo* adverse effects were defined as a 30% change from baseline in one of the following parameters tidal volume (VT), respiratory rate (BPM), time of break (TB) or time of pause (TP). LOAEC was defined as the concentration where changes in one or more of these parameters was observed. For three of the PPPs a LOAEC could not be determined, as exposure to even the highest generated aerosol concentration did not change any of these parameters, this concentration was designated the NOAEC (no observed adverse effect concentration). The aerosol concentration during the LOAEC (or NOAEC) experiments as measured by filter sampling were combined with the time of exposure, VT and BPM to estimate the deposited dose. The estimated dose and affected parameter(s) are collected in the second and third column in table 4.

When the same PPPs were tested for LS function inhibition *in vitro*, adverse reaction was defined as an elevation in minimum surface tension to >10 mN/m. The inhibitory, or maximum exposed dose (if the product did not inhibit function) was measured using a quartz crystal microbalance (QCM) placed in the exposure chamber. The results have been collected in column 4, 5 and 6 in table 4.

To determine if the *in vitro* test could predict the toxicity observed in the animals, we assumed that the *in vivo* data represents the true result (table 4). Thus a true positive or negative prediction (TPos or TNeg) means that the *in vitro* result predicted the *in vivo* outcome. Conversely if the *in vitro* result did not predict the *in vivo* outcome these were considered false positive or negatives (FPos or FNeg).

TABLE 4. The effect of PPPs on breathing patterns of exposed mice and lung surfactant function.

	In vivo		***In vitro***			**Correlation**
	Estimated deposited dose, µg/mg LS	affected parameter	inhibition	inhibitory dose, µg/mg LS	maximum dose[$], µg/mg LS	
A	4	VT, TP	yes	17		TPos+
B	24	VT, BPM, TP, TB	yes	7		TPos-
C	34	TB	no		4	FNeg-
D	<0.4 *	TB	yes	65		TPos+
E	4	VT, TP, TB**	yes	42		TPos+
F	21	TB	no		50	FNeg+
G	6	VT	no		73	FNeg+
H	21	TB	yes	7		TPos-
I	12	none	no		5	TNeg-
J	10	none	no		12	TNeg+
K	28	none	yes	20		FPos-

* the deposition on the filter was below detection limit, ** two of five animals experienced rapid reduction in tidal volume and had to be removed from exposure and killed, $ the maximum deposited dose is taken from the plateau formed after 5 min of aerosolisation *in vitro*, TP: time of pause, TB: time of break, VT: tidal volume, TPos: true positive, TNeg: true negative, FPos: false positive, FNeg: false negative.

We also evaluated if the *in vitro* test was done at the same estimated dose as *in vivo*. A "+" in the last column in table 4 indicates that the inhibitory or maximum dose tested *in vitro* was higher than the LOAEL obtained from the *in vivo* experiment. Conversely a "-" indicates that the *in vitro* test was performed at a lower concentration than in the *in vivo* test.

All AI and co-formulants found in the PPP MSDSs were run through the Danish QSAR database for respiratory sensitization and the RespiraTox QSAR. The results are collected in table 5.

TABLE 5. Predictions made by two QSAR models, the Danish QSAR database and RespiraTox.

Chemical name	**CAS number**	**Found in product**	**Danish QSAR database**	**RespiraTox**
Active ingredients			**Respiratory Sensitisation in Humans**	**prediction**
Azoxystrobin	131860-33-8	A	INC_OUT	irritant
Boscalid	188425-85-6	B, C, K	INC_OUT	irritant
Epoxiconazole	133855-98-8	B	INC_OUT	irritant
Pyraclostrobin	175013-18-0	D, K	INC_OUT	irritant
Cycloxydim	99434-58-9	E	-	irritant
Glyphosate	1071-83-6	F	INC_OUT	irritant
Metconazole	125116-23-6	G	INC_OUT	irritant
Kresoxim-methyl	143390-89-0	H	INC_OUT	-
Propyzamide	23950-58-5	I	POS_IN	irritant
Pyrimethanil	53112-28-0	J	INC_OUT	irritant
Co-formulants				
Docusat sodium	577-11-7	E	NEG_IN	-
Calcium dodecylbenzenesulphonate	26264-06-2	D	NEG_OUT	-
2-ethylhexan-1-ol	104-76-7	D	NEG_IN	irritant
Sodium diisobutylnaphthalenesulphonate	27213-90-7	K	INC_OUT	-
Naphthalen	91-20-3	D, E	NEG_OUT	irritant
1,2-benzisothiazol-3(2H)-on	2634-33-5	B, J	INC_OUT	irritant
Propan-1,2-diol	57-55-6	A, B, J	POS_IN	irritant
Diethylenglycol	111-46-6	G	POS_OUT	non-irritant
1,2-Ethandiol	107-21-1	I	POS_IN	non-irritant
Cellulose	9004-34-6	B	-	irritant

"-" QSAR could not make a prediction, prediction can be positive (POS), negative (NEG) or inconclusive (INC), in addition to being in the domain of the QSAR (_IN) or out of domain (_OUT)

TABLE 6. Collection of results for each AI

AI	EU registration	Literature	Product	*In vitro*	*In vivo*
Azoxystrobin	LC_{50} inhalation: >700 mg/m^3 air (particle size < 2 µm) [33].	In a report from WHO evaluating azoxystrobin, the LC_{50} was found to be 698 mg/m^3 for female and 962 mg/m^3 for male rats. The rats developed respiratory sign of toxicity beginning at 500 mg/m^3, during and up to 4 days after exposure [34].	A (only AI)	Product A: inhibition after 3 min exposure, at 17 µg/ mg LS.	At aerosol concentrations of 1.7 mg/m^3 of product A for 30 min (LOAEC), 2 of 7 animals experienced pulmonary irritation (elongated TP). The same animals had a gradual reduction in tidal volume. In the concentration-range experiment (where the infusion rate of the product was higher, but the concentration not determined), exposure likewise resulted in increase in TP.
Boscalid	LC_{50} inhalation: >6700 mg/m^3 air (nose-only dust exposure) [35].	When boscalid was tested at 6700 mg/m^3 there were no deaths, but rats experienced respiratory signs of toxicity [36].	B (with epoxiconazole)	Product B: inhibition after 1 min exposure, at 24 µg/ mg LS.	A 60 min exposure at 9.5 mg/m^3 (LOAEC) resulted in an immediate increase in respiratory rate, TB and TP, and reduction in VT, the strongest effect was sensory irritation (TB). During the concentration-range experiment only TP was affected.
			C (only AI)	Product C: did not inhibit function after 5 min exposure, final exposure was 4 µg/ mg LS.	Exposure to product C at 15.2 mg/m^3 for 60 min (LOAEC) resulted in an initial increase in respiratory rate, TP and TB, and a decreased VT. After 20 min the values had returned to baseline, except for TB that remained increased, indicating that the main effect was sensory irritation. During the concentration-range experiment, the effect was less pronounced.
			K (with pyraclostrobin)	Product K: inhibited LS function after 4 min of exposure, at 20 µg/ mg LS.	When mice were exposed to product K for 60 min at 6.3 mg/m^3 this did not change the respiration (NOAEC).
Epoxiconazole	LC_{50} inhalation: No info in registration.	Low acute toxicity by inhalation in rats (LC_{50} >5300 mg/m^3) [37].	B (with boscalid)	See above (product B).	See above (product B).
Pyraclostrobin	LC_{50} inhalation: 690 mg/m^3 [38].		D (only AI)	Product D: inhibited LS function after 40 seconds of exposure, at 65 µg/ mg LS.	The filter sample collected during the LOAEC experiment for product D was below the detection limit, thus the aerosol concentration was <0.2 mg/m^3. During the 60 min exposure there was an increase in TB, indicating sensory irritation. During the concentration-range experiments, the product caused increase in breathing rate, TP and TB and reduced tidal volume, however these changes returned to baseline levels when the mice were given clean air to breathe.
			K (together with boscalid)	See above (product K).	See above (product K)
Cycloxydim	LC_{50} inhalation: No info in registration.	LC_{50}>5280 mg/m^3 [39].	E (only AI)	Product E: inhibited LS function after 50 seconds of exposure, at 42 µg/ mg LS.	Exposure to product E for 60 min at an aerosol concentration of 0.8 mg/m^3 (LOAEC) caused reduction in tidal volume and large increase in TP and TB, and 2 of the 5 exposed animals had to be killed during the exposure due to very fast and severe reduction

					in tidal volume. During concentration-range similar changes were seen at high concentrations.
Glyphosate	LC_{50} inhalation: >5000 mg/m^3 air (4-hour exposure) [40].	A study with human volunteers simulate heavy residential spraying of glyphosate showed that glyphosate is eliminated from the body within 24 h, and skin absorption results in more exposure than inhalation [41]. Nursery workers that routinely used glyphosate had no detectable metabolites in their urine samples [42].	F (only AI)	Product F: no inhibition after 5 min exposure, at a maximum of 50 µg/ mg LS.	When mice were exposed to product F for 50 min at an aerosol concentration of 7.9 mg/m^3 there was an increase in TB, indicating sensory irritation.
Metconazole	LC_{50} inhalation: No info in registration.	LC_{50} inhalation: >5600 mg/m^3 air [43].	G (only AI)	Product G: no inhibition after 5 min exposure, at a maximum of 73 µg/ mg LS.	When mice were exposed to 9.6 mg/m^3 of product G for 15 minutes, there was a decrease in the tidal volume (LOAEC). When mice were exposed to higher concentrations during concentration-range finding experiments, there was also an increase in TP, indicating pulmonary irritation. Both the reduction in tidal volume at LOAEC, and the TP increase was reversed during recovery.
Kresoxim-methyl	LC_{50} inhalation: >5600 mg/m^3 [44].	A risk assessment evaluation of spraying [45] kresoxim- methyl in an apple orchard and the mixing and loading [46] prior to spraying concluded that the operations constitutes minimal risk to the workers.	H (only AI)	Product H: inhibited LS function after 5 min of exposure at 7 µg/ mg LS.	When mice were exposed to an aerosol concentration of 10.3 mg/m^3 of product H for 60 min (LOAEC), they experienced an increase in TB indicating that the product caused sensory irritation. During concertation-range experiments the increase in TB was accompanied by a drop in tidal volume.
Propyza-mide	LC_{50} inhalation: >2100 mg/m^3 air [47].		I (only AI)	Product I: no inhibition after 5 min exposure, at a maximum of 5 µg/ mg LS.	When mice were exposed to 3.3 mg/m^3 aerosol of product I for 60 min, no changes were seen on the breathing patterns of exposed mice (NOAEC).
Pyrimethanil	LC_{50} inhalation: No info in registration.	Giffin *et al* [48] found association between levels of pyrimethanil sampled in the air and the metabolite concentrations in the urine. The study shows that inhalation is a major route of exposure. LC_{50} inhalation: >1980 mg/m^3; max. attainable concentration [49].	J (sole AI)	Product J: no inhibition after 5 min exposure, at a maximum of 12 µg/ mg LS.	When mice were exposed to product J at 3.2 mg/m^3 for 40 min (NOAEC), no changes were observed in the breathing patterns of exposed mice.

7.1 Collection of data for co-formulants

We used the publicly available MSDS for each product to collect information on the chemicals present in the products in addition to the active ingredient (Table 3). As only chemicals that are potentially hazardous to human health or the environment appear in the MSDS, the full chemical composition of the products could often not be identified. The summed percentages of constituents reported in the MSDS accounted for 49% (product F) to 100% of the composition (product G).

Two of the co-formulants were predicted to be respiratory sensitizers in humans by the Danish QSAR database (table 4), propan-1,2-diol and 1,2-ethandiol. However, they were not classified as such under CLP. Propan-1,2-diol was proposed to have a harmonized classification under CLP to be irritating to the respiratory tract (STOT SE 3, triggering H335 "may cause respiratory irritation"), but the committee for risk assessment (RAC) found that the evidence was insufficient for this classification [50]. The two following studies are examples of how the data points in opposite directions. In humans inhalation exposure to propan-1,2-diol was found to reduce FEV1[3], induce throat symptoms and development of cough in some individual test persons after 1 min exposure to 309 mg/m^3 [51], whereas a later study, also in humans, did not find any effect after 4h exposure at 200 mg/m^3 [52]. In the ECHA dossier for 1,2-ethandiol one inhalation study in rats is mentioned, however no deaths or respiratory signs of toxicity were recorded at 2,500 mg/m^3.

2-ethylhexan-1-ol is classified under CLP, and labelled with the hazard statements H335: May cause respiratory irritation and H332: Harmful if inhaled. In the ECHA dossier two inhalation experiments were performed, with the substance as a vapour or as an aerosol. Exposure as a mix of aerosols and vapour resulted in an LC_{50} of 5300 mg/m^3, the animals had respiratory signs of toxicity and died during exposure. However when the substance was tested only as a vapour there were neither deaths nor respiratory signs of toxicity (>LC_{50} 890 mg/m^3), the concentration could not be further increased during test as a vapour.

Napthalene inhalation in humans causes headache, confusion, eye irritation, nausea, and profuse perspiration with vomiting, optic neuritis, haematuria, and oedema (at unknown concentration levels) [53]. In the ECHA dossier, naphthalene was tested at the highest possible aerosol concentration (400 mg/m^3), at this concentration it caused no deaths of the animals, and the only respiratory sign of toxicity was "mouth breathing" at the day of exposure.

When diethyleneglycol was studied in mice it caused sensory irritation, measured as a decrease in respiratory rate. The RD_{50} (reduction of respiratory rate to 50% of baseline) is used to rank substances, and the RD_{50} of diethyleneglycol was found to be 11,600 mg/m^3 [54].

Sodium diisobutylnaphthalenesulphonate is classified in CLP as Inhalation Acute Tox. 4 triggering the hazard statement "H302+H332: Harmful if swallowed or if inhaled", the LC_{50} was reported to be >9330 mg/m^3. At this concentration the rats experienced laboured breathing.

Docusat sodium, 1,2-benzisothiazol-3(2H)-on, and calcium dodecylbenzenesulphonate have ECHA dossiers, but with no information on inhalation exposure or classification relating to inhalation toxicity. Cellulose does not have an ECHA dossier.

7.2 Aerosol measurements

The particles generated during the *in vivo* exposures were for most PPPs bimodal. One particle mode was <100 nm in diameter with a second mode observed in the range of 0.5-1 µm. As

[3] FEV1 is the amount of air that can be exhaled during the first second of a forced breath after a maximum inhalation.

most of the particles were in the respirable range, we assumed that all particles could be inhaled for the purpose of estimating the dose deposited in the alveoli. The filter sampling done during the exposure was used to estimate the alveolar deposited dose during exposure. Aerosol measurements can be seen in supplementary figure 1.

8. Discussion

We tested the effect of eleven PPPs on LS function and on breathing patterns of exposed mice. We also used two inhalation relevant QSARs to predict the effects of the AIs and co-formulants.

The following discussion highlights two dilemmas/paradigm shifts that are emphasised in our collected and generated data for the AIs and PPPs studied in this project.

1. The data used up until now for regulation of chemicals, including AIs and PPPs, are based on inhalation experiments where the reason for the endpoint (death of the animal) is not determined. This endpoint is not possible to recreate/replace without whole animals. Therefore, we have performed animal experiments, where the endpoint is effects on the portal of entry, i.e. the lungs, and specifically, changes in breathing pattern.
2. Alternative methods can only recreate part of, or single organs. The move away from death to local effects allow results from alternative methods, e.g. inhibition of LS function, to be compared to the results that stem from the effects on the lungs *in vivo*. However, as the lung is complex (and forms part of an intact organism) the different effects can still arise due to many different mechanisms of toxicity.

This leaves two (not mutually exclusive) avenues for replacement. The first approach aims to replace animal experiments by showing that the *in vitro* results are predictive of the effects observed *in vivo* in regulatory studies, or as in the case of this project effects on the lungs. The comparison can be used to build confidence in that regulation based on *in vitro* results will protect human health as well as if animal experiments were performed. The second approach is to understand the underlying mechanisms of toxicity that can be triggered by inhalation, and then use this knowledge to develop specific assays to test for this mechanism *in vitro*. In this project we have combined the two (performing animal tests while measuring effects on the lungs, and testing *in vitro*), and point to what is needed to achieve the latter (understanding lung toxicity for full replacement).

With some exceptions, most PPPs and AIs have to be tested for acute inhalation toxicity according to the accepted OECD TGs [1, 2]. In these studies, the exposure concentrations are pre-set, and the endpoint is death (or evident signs of toxicity) during the 14 days of observation post-exposure. The resulting LC_{50} values are much higher (up to 1000 fold) than any of the concentrations that was found to change respiration patterns in this study. For example, when azoxystrobin was evaluated as an AI within the EU, the LC_{50} was 700 mg/m^3 [33]. In contrast, we found a LOAEC at 1.7 mg/m^3 due to pulmonary irritation, when we tested product A containing 25% of the AI (albeit together with other substances). This underlines the large qualitative difference in the endpoint of death compared to the respiratory changes. The changes in breathing patterns are likely not fatal, but nevertheless indicative of respiratory toxicity.

For the OECD TG studies, it has been estimated that adverse effects on the lungs precede systemic effects for up to 90% of the tested substances [55]. To determine the LC50, the chemical has to damage the lungs until the point of fatality or induce other lethal systemic toxicity. In the OECD TG studies, altered breathing patterns are noted as clinical signs of exposure. The TG433 [5] is a refinement of TG403 [4] and TG436 [6]. In TG433 “evident signs of toxicity” is used as the endpoint for the individual animal, whereas death is the endpoint in TG403 and 436. The work that lead to acceptance of TG433 included collection of clinical data

from TG403 and 436 experiments performed for regulation. Clinical signs were extracted at the second highest test level and evaluated for their predictability of death at the next exposure concentration [7]. Among these, evident signs of toxicity included changes in respiration, i.e. “irregular respiration” (in 42% of the cases), “laboured respiration” (16%), “congested respiration” (5%) and “noisy respiration” (0.4%). Irregular respiration had a positive predictive value of death at the next level of exposure of 89% [7].

The interpretation of altered breathing patterns, noted during guideline studies and observed during monitoring by plethysmography, is challenging. This is because; 1) in guideline studies changes are observed cage side and as such are subjective to the observer. This makes it difficult to interpret if different chemicals have similar effects, as the observer describes the observations in their own words, 2) changes in breathing that can be observed by eye have to be pronounced to be noticed, e.g. mice breathe at a rate of 250-300, and rats at 60-80 breaths per minute. 3) changes in breathing patterns are gradual compared to the dichotomous live/dead endpoint. Interpretation therefore requires a definition of what is considered an adverse change in breathing pattern

The changes in respiration observed when the animals were exposed in whole body plethysmograhs are mild compared to cage side observed changes. Mild effects on breathing have been used to study toxicity previously. To this end, Alexeeff et al [56] studied mild effects in experimental animals or in exposed humans to set acute (1 hour) inhalation reference exposure levels (RELs) for hazardous air-born substances. Mild effects included cough urge, respiratory irritation and upper airway symptoms. The Alarie assay, where changes in breathing are monitored by plethysmographs, uses a cut off value of 50% reduction in respiratory rate (RD_{50}) [21]. This RD_{50} has been used to set threshold limit values for the working environment based on sensory irritation [57]. In the present study, we use a similar setup as the Alarie assay, however we did not see severe reductions in breathing rates, likely because; 1) the Alarie assay is primarily used for volatile organic chemicals (our test substances had low volatility), and 2) we terminated exposure due to changes in VT, and not breathing rate.

In an attempt to clarify how inhalation toxicity studies could be used more efficiently, Arts et al [58] suggested to use 20% decrease in breathing rate as a relevant change in the context of using data from animal experiments to evaluate non-lethal assessment of chemical exposure. We defined changes in the parameters of breathing rate, tidal volume, time of pause, and time of break at ≥30% compared to baseline as severe, as these could clearly be distinguished from the changes occurring due to restraining the animals during the experiment. The changes in breathing parameters are difficult to relate to the LC_{50} values set on the basis of OECD TG studies. It is also very likely a less severe change than those reported cage side during guideline studies. However, a 30% decrease in these breathing parameters would be unacceptable in exposed humans. Six of the tested PPPs increased the time of break at the LOAEC indicating that the product irritated the upper airways. Three of the products increased the time of pause indicating that the products initiated pulmonary irritation, and three products reduced the tidal volume of the exposed animals.

In vitro, we measured the effect of the PPPs on LS function. Inhibition of LS function can lead to severe pathology of the lungs, as outlined in the AOP (Fig. 1), LS inhibition starts a cascade that ultimately decreases lung function. Decreased lung function can also be triggered by other conditions such as inflammation or direct damage to the lung cells. Reduced LS function has been associated with increased mortality in the acute respiratory distress syndrome in humans. This is however a complex disease with damage occurring at many levels in the lung, hence inhibition of LS function cannot be separated from other pathologies [59]. In previous projects, we have tested a group of spray consumer products, i.e. impregnation products. These products frequently cause adverse effects to the lungs after inhalation in humans. We used the same set-up as in this project for testing of impregnation products, i.e. both testing for

LS inhibition *in vitro* and monitoring of breathing patterns *in vivo* [17]. Impregnation products are, as PPPs, formulated as mixtures of several substances. When we tested impregnation products, the most prevalent *in vivo* effect was rapid decrease in tidal volume, followed by death of the animal, unless exposure was terminated immediately [17]. A common ingredient in many of the toxic impregnation products was hydrocarbon/petroleum solvents. In the present study, PPP products D and E contained hydrocarbon solvents (C10 hydrocarbons). These two products caused by far the most pronounced reaction in the exposed mice, and at the lowest infusion rates of the PPPs tested (supplementary table 2). Product D lead to sensory irritation in the LOAEC experiment. Likewise, pulmonary irritation and increased breathing rate and decreased tidal volume were observed in the concentration-range experiments. For product E, both sensory and pulmonary irritation were observed alongside reduction in tidal volume and increase in breathing rate in the LOAEC experiment. In addition, exposure lead to a sudden drop in tidal volume so that mice had to be removed from exposure and killed. When hydrocarbon/petroleum based impregnation products were tested in the same set-up, the NOAEC infusion rates ranged between 0.27 µl/min and 8 µl/min compared to 0.3 and 1.5 µl/min in the LOAEC experiments for product D and E, respectively (supplementary table 2). This suggests that at least part of the respiratory effects occurred due to the solvents. To elucidate if also other components of the PPPs contributed to the effect, e.g. the AIs, each component would have to be tested separately.

Using the effects observed in the animal experiments as the true value, the following predictive matrix can be made when combined with predictions made from the *in vitro* data.

TABLE 7. The predictiveness of the *in vitro* data when the outcome of animal testing was defined as true.

		***In vitro* inhibition**			
		yes	no		
***In vivo* change in breathing pattern**	yes	5	3	positive predictive value	0.83
	no	1	2	negative predictive values	0.4
		Sensitivity 0.65	Specificity 0.66	Accuracy 0.64	

The sensitivity and the specificity is quite low when the LS function inhibition is used as predictor of changes in breathing pattern of the exposed mice (0.65 and 0.66 respectively, table 7), with an overall accuracy of 64%. The predictivness for other products or chemicals have been shown be much higher, e.g. the overall accuracy of using LS inhibition to predict changes in breathing patterns for impregnation products, inhaled pharmaceuticals and single chemicals was 86%, 100% and 81% respectively (table 1). The lower predictivness of LS inhibition by PPPs can have several explanations: 1) only one in *in vitro* test is used to predict effects that can stem from several biological entities in the lung, 2) all changes to breathing patterns are considered relevant, even if they might have different triggers. In addition AIs undergo rigorous regulation and testing prior to approval, and PPP use is restricted to professionals.

Testing PPPs in a battery of lung related *in vitro* tests would likely have improved the predictiveness. In addition to LS function, tests that measure e.g. effects on epithelial barrier function, whether the chemical penetrates the barrier, cytotoxicity, and if the compounds trigger nerves in the respiratory system are likely candidates for this battery. *In vitro* tests with relevance for these endpoints are under development or already for sale. The latter include models that cover the upper- and small airways from e.g. Epitelix or MatTek and an alveolar model

from Alveolix. LS function cannot be tested in these cell models, thus these methods complement each other. The specific models that should be incorporated in a battery of tests are yet to be determined. There are however projects both in regulatory bodies (e.g. US EPA), in industry (e.g. Unilever) and in academia that are trying to establish which should be included. An approach to use alternative methods has already been explored for the AI chlorothalonil in the US. In 2014, Syngenta suggested using a cell model, MucilAir, to test chlorothalonil. The US regulation requires a 90 day inhalation study, but chlorothalonil was already identified as an airway irritant [60]. Syngenta argued that the information from the animal study would not provide additional information. Rather, the animals would be exposed to a known irritant. The US EPA are still evaluating if the data from the cell model can be used instead of the 90 day inhalation test [60]. If the data is deemed suitable, this will open the door for use of alternative methods in regulation. This highlights that alternative tests are presently available, but that regulatory confidence in these methods have to be built, both in the EU and in the rest of the world.

The relatively low predictiveness could also be due to the comparison of inhibition of LS function with “change in breathing pattern” in animals. The latter depends on the specific effects the chemical has on the lungs, and this may differ from chemical to chemical. As an example, time of break elongates because of stimulation of the trigeminal nerve endings in the upper respiratory tract. Time of pause elongates when the vagal nerve endings at the alveolar level are activated [22]. If LS function is inhibited, it is probably more likely to be associated with pulmonary irritation than effects of the upper airways. Also changes in tidal volume and breathing rate could likely be linked to inhibition of LS function, as the latter would lead to alveolar collapse (and reduced tidal volume) and a compensatory increase in breathing rate. Changes in time of break could therefore be considered negative relative to correlation between *in vitro* and *in vivo* outcomes. This would change the predictiveness of LS inhibition so that sensitivity increases to 0.8, and specificity to 0.5, i.e. the *in vitro* test predicts most of the *in vivo* effects, but over-predicts products that do not have an *in vivo* effect (high rate of false positives). This again shows the need to combine results from a battery of *in vitro* tests that measures different effects on the lung.

A review of epidemiological studies suggest there is a link between occupational exposure, and in the general population, to PPPs and respiratory effects [61, 62]. However, the literature that these reviews are based on have not been segregated by geographical location. As the regulation of AIs, PPPs and their use differ within and outside the EU, it is difficult to draw conclusions related to the use of PPPs in the EU based on studies of populations that could have been exposed to AIs/PPPs not marketed in the EU.

To aid interpretation of the results gained in this project, we searched for what was already known about the chemicals in the PPPs relative to effects of inhalation exposure. The ICE database curates the data from the dossiers handed in during registration of the products with the US EPA, albeit lack of information on co-formulants is a major drawback. Hence, a search returns a largely useless collection of LC_{50} values for products containing the same AI. Searches in the PubMed database for publications related to the effect of the PPP ingredients on the lungs, proved that there are large knowledge gaps.

We used the registration information for the different AIs to extract information on inhalation experiments and LC_{50} values. Likewise, we used the REACH database to obtain similar information for the co-formulants. The LC_{50} values are so much higher than the aerosol concentrations tested in the present project that they were not informative. However, for a few of the components of the PPPs, the experiments were described in sufficient detail for signs of airway toxicity to be extracted, and we have previously shown that LS inhibition is predictive of this endpoint [16]. However, this highlights a separate problem with products that are formu-

lated and sold. As the MSDS sheets that are supplied by the producers only contain information on single constituents that are known to be harmful to human health or the environment, we do not know the complete composition of the products. As it is known that different chemicals can influence each others toxicity, particularly when formulated as PPPs [10], testing separate chemicals does not provide enough information to properly evaluate the toxicity of the products.

There is a large potential in developing a QSAR that can either predict effects used presently for regulation, i.e. leading to death (unlikely) or adverse effects on the airways. A QSAR model that is very sensitive, i.e. the model predicts if a chemical will cause adverse effect of the airways with high certainty, should be used as screening tool to avoid testing of toxic chemicals. However, if the QSAR has high specificity, i.e. it predicts that the chemical does not have an adverse effect with high certainty, this would prompt no further testing. We used two QSARs for prediction of lungs effects. The RespiraTox QSAR was built to predict respiratory irritation. This endpoint is likely closely linked to the effects resulting from inhibition of LS function (AOP 302, [20]). However, this QSAR presently has major limitations. The predictions are “worst case”, therefore the QSAR predicted most AIs and co-formulants (18/20) to be airway irritants. If the method undergoes refinement it might become useful, however at present it does not add to the hazard assessment evaluation due to the large number of false positives relative to the present results. The second QSAR, predicts respiratory sensitization (as part of the Danish QSAR database), an immunological effect in the lung. This effect requires previous inhalation of the compound, and leads to a reaction in the airways at subsequent exposure. This effect therefore differs qualitatively from the one tested for in this project. It is at present unknown whether there is a link between respiratory sensitisation and LS function inhibition. However, in an overall assessment of the effect of chemicals on the respiratory system, this QSAR could potentially be used as a screening tool. In the present context, the model predicted one AI and two co-formulants as respiratory sensitizers, and two co-formulants as not respiratory sensitizers. The predictions for the remaining compounds were out of the domain of the QSAR.

9. Conclusion

The results from the present project show that 1) some PPPs exert acute effects on respiration at aerosol concentrations much lower than the LC_{50} values determined by OECD TG studies. 2) The literature on lung effects of AIs and co-formulants has large knowledge gaps. Most AIs and co-formulants have been tested for acute inhalation toxicity in TG studies (table 6), but in most cases LC_{50} is reported, rather than the levels at which effects on the lungs occur. 3) The *in vitro* method measuring inhibition of LS function cannot stand alone as a prediction tool for lung toxicity as lethality cannot be recreated *in vitro*, but should form part of a battery of tests testing different endpoints of lung toxicity. 4) The predictivness of LS function inhibition for changes in breathing patterns for PPPs is lower than for other substance groups (table 1 and table 7), the reason for this is not determined.

The move away from animal testing for acute inhalation toxicity and calculation of LC_{50} requires a paradigm shift, from reliance on death as the endpoint to focus on less severe effects at the portal of entry (the lungs). Effects on the LS function can be tested *in vitro*, and will, in combination with outcomes from several tests of other aspects of lung function, give a high level of confidence in the predictiveness relative to overall toxicity to the lungs. The tests to be included in such a battery are yet to be determined, but this and other projects show that the LS function testing is important, particularly because it cannot be recreated in other *in vitro* tests such as those relying on cell cultures. Future work to complement this project would be to 1) test the individual substances in the PPPs that had an effect either *in vitro* or *in vivo* to pinpoint which is causing the effects, e.g. to feed in to a QSAR model for “lung surfactant function leading to decreased lung function”, and 2) testing of the PPPs and/or their single constituents in other *in vitro* assays to see if this improves the prediction of the *in vivo* effects further.

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11. Supplementary material

Appendix 1.

In supplementary table 1 the number of animals that were used to test each PPP has been collected, the number varies because of the variation in reaction to the exposure between the animals and, because for some more experiments had to be performed to find the LOEAC.

SUPPLEMENARY TABLE 1. Number of animals used to test each product

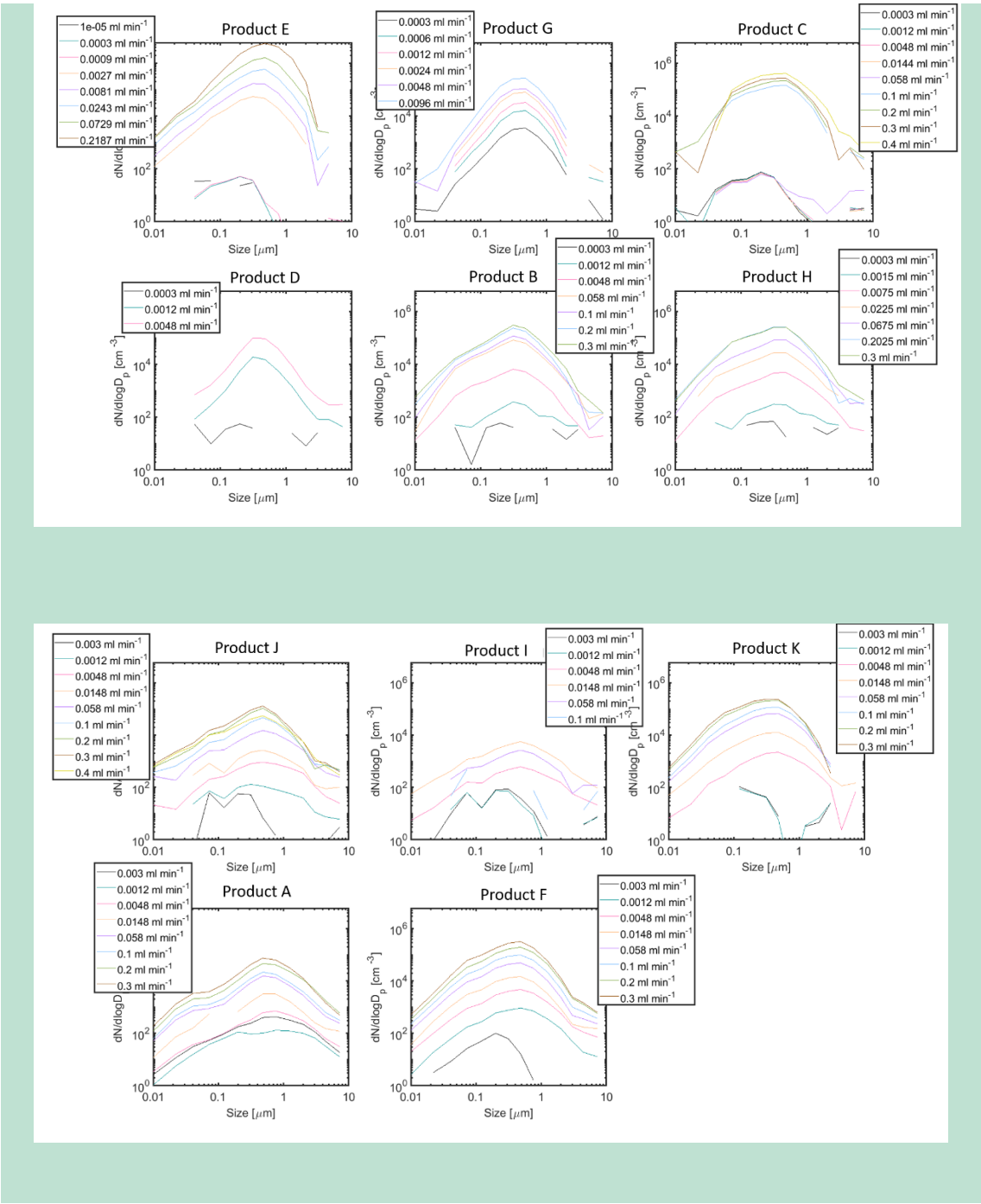
Product	# of mice
A	18
B	14
C	21
D	17
E	31
F	16
G	21
H	15
I	13
J	12
K	13

In supplementary table 2 the infusion rate and concentration of the test solutions are collected for the *in vivo* and *in vitro* experiments. The concentrations depend on the aerosolisastion of the PPP. If the PPP was to concentrated it clogged up the aerosolisation system, thus the test concentration is the highest that could be aerososlised. The infusion rates *in vivo* are low if there was an effect of breathing patterns (the lowest infusion rate giving an effect), and high if there was no effect *in vivo*. *In vitro* we aimed for an infusion rate of 1 ml/min, for some this was not possible, and the infusion rate was lowered until it became possible to aersolise for 5 min.

SUPPLEMENTARY TABLE 2. Test concentration and infusion rate *in vitro* and *in vivo*

Product	**Dilution *in vivo***	**Infusion rate and time at LOAEC or NOEAC**	**Dilutions *in vitro***	**Infusion rate and at inhibition of LS function**
A	50%	0.1 ml/min, 30 min	50%	1 ml/min, 3 min

B	50%	0.2 ml/min, 60 min	50%	1 ml/min, 1 min
C	10%	0.3 ml/min, 60 min	2%	1 ml/min, -
D	No dilution	0.0003 ml/min, 60 min	No dilution	0.8 ml/min, 40 sec
E	No dilution	0.0015 ml/min,60 min	No dilution	0.5 ml/min, 50 sec
F	10%	0.2 ml/min, 60 min	50%	1 ml/min, -
G	No dilution	0.01 ml/min, 15 min	No dilution	0.7 ml/min, -
H	10%	0.3 ml/min, 60 min	10%	0.8 ml/min, 5 min
I	50%	0.2 ml/min, 60 min	50%	0.8 ml/min, -
J	50%	0.3 ml/min, 40 min	50%	1 ml/min, -
K	50%	0.2 ml/min, 60 min	2%	1 ml/min, 4 min



SUPPLEMENTARY FIGURE 1. ELPI measurements of aerosols generated at different infusion rates.

Expansion of table 2 in manuscript. Co-formulant grouping, name and CAS number from MSDS

Wetting agents:

- Alcohols, C16-18, ethoxylated propoxylated, 68002-96-0
- Alcohols, C16-18 ethoxylated, 68439-49-6
- Alcohols, C9-11, ethoxylated, 68439-46-3
- Naphthalenesulfonic acid sodium salt, 9008-63-3

Emulsifiers

- Docusate sodium, 577-11-7
- calciumdodecylbenzensulfonat, 26264-06-2
- 2-ethylhexan-1-ol, 104-76-7
- Oxirane, methyl-, polymer with oxirane, monoisotridecyl ether, block, 196823-11-7
- Phenolsulfonsyre-formaldehyd-polykondensat som natriumsalt
- Reinigungsmittel AG 6202
- Lignin, alkali, reaction products with formaldehyde and sodium bisulfite, 68512-35-6
- Sodium diisobutylnaphthalensulfonat, 27213-90-7

Solvents

- Hydrocarbons, C10, aromatics, <1% naphthalene, 64742-94-5
- naphthalen, 91-20-3

Preservative

- 1,2-benzisothiazol-3(2H)-on, 2634-33-5

Anti freeze

- propan-1,2-diol, 57-55-6
- diethylenglycol, 111-46-6
- 1,2-Ethandiol, 107-21-1

Thickener

- cellulose, 9004-34-6
- ammonium sulphate, 7783-20-2
- Sodium sulphate, 7757-82-6
- Silica gel, precipitated, crystalline free, 112926-00-8

SUPPLEMENTARY TABLE 3. Data retrieved from the ICE database on “Acute Inhalation Toxicity Assay (*in vivo*)” of different AIs.

AI	ICE
Azoxystrobin	11 products Mixed with other AIs (10), respiratory toxicity shortly after exposure (8)
Boscalid	4 products Mixed with other AIs (4), respiratory toxicity (2)
Epoxiconazole	No info
Pyraclostrobin	9 products Mixed with other AIs (6), respiratory toxicity (9)
Cycloxydim	No info
Glyphosate	10 products Mixed with other AIs (2), respiratory toxicity for 8 products where glyphosate was only AI
Metconazole	5 products Mixed with other AIs (3), respiratory toxicity (2)
Kresoxim-methyl	No info
Propyzamide	No info
Pyrimethanil	1 product Mixed with other AIs (0) respiratory toxicity (0)

Inhibition of lung surfactant function as an alternative method to predict lung toxicity following exposure to plant protection products

Currently acute inhalation toxicity is a required test for pesticide active ingredients (AIs) and formulated plant protection products (PPPs). As there are no accepted alternative methods for testing of this endpoint, animal experiments are required. The outcome of the test used for regulating the chemicals is “lethal concentration 50”, the concentration that will kill 50% of the exposed animals (LC50). In this study, we investigated an alternative method for determining the effect of inhaled substances on the lungs. We studied 11 PPPs for their ability to inhibit lung surfactant (LS) function in vitro, and subsequently evaluated if this predicted changes in breathing patterns of exposed mice. Six of the eleven PPPs inhibited LS function, and eight changed the breathing pattern of exposed mice. Most of these caused changes indicative of sensory irritation (6), three caused changes indicative of pulmonary irritation and two caused a reduction in tidal volume (one product cause all three changes). The results from the in vitro inhibition of LS function predicted changes in respiration of exposed mice with a sensitivity of 65% and a specificity of 66%. Testing the effect of inhaled substances on LS function is not a method accepted as an alternative to animal testing in regulatory guidelines, however the test can be used to test the molecular initiating event in an adverse outcome pathway currently under evaluation by the OECD.

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