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# Contents

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Original Research Article

# Toxicological evaluation of Ultrapure and Potent Tannic Acid (UPPTA) by inhalation exposure

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**ABSTRACT:** The therapeutic options for coronal virus infections are limited. As SARS-CoV-2 directly targets the lungs and causes lung damage, treatment of COVID-19 with inhalants may offer more advantages over oral administration. Inhaled drug delivery provides a higher drug concentration in the target organ, where SARS-CoV-2 proliferates. In this study, we evaluated the potential systemic toxicity, relevant target organ toxicity, and toxicokinetics of Airnecflu®, Ultrapure, and Potent Tannic Acid (UPPTA) by metered-dose inhaler (MDI) inhalation to rodent and canine species once a day for 2 consecutive weeks. We further investigated the reversibility of the toxicity following a 3-week recovery period. No mortality related to the test article was observed in all the dose groups. Neither abnormalities related to the test article nor toxicologically significant changes were observed in both rodent and canine studies. In pathological examination, alveolar macrophage aggregation, perivascular/interstitial/alveolar inflammatory cell infiltration, and alveolar/bronchial epithelium hyperplasia were noted in the lung with bronchial involvement. However, after a 3-week recovery period, a substantial recovery was observed. There is limited systemic exposure to the inhalation administration. Therefore, inhalation of Airnecflu® UPPTA is safe to administer for respiratory disorders like COVID-19.

***KEYWORDS:*** COVID-19; SARS-CoV-2; metered-dose inhaler; inhaled toxicity; pulmonary pathology

## 1. Introduction

Tannic acids, a group of tannins enriched in several species of sumac and Aleppo oak, are naturally occurring hydrolysable polyphenol compounds with galloyl moieties expanding from a central glucose moiety. The structures of tannic acids are composed of 1 to 10 units of galloyl moieties per molecule. Throughout human history, tannic acids have been used as (part of) herbal medicine worldwide for thousands of years. For example, tannic acids have been used to treat diarrhea, bleeding, coughing, and detoxification in China[1].

The study of the anti-viral activity of tannic acids can be traced back more than 70 years. Green first reported that tannic acids can inhibit influenza A virus multiplication and hemagglutination[2]. More recently, tannic acids extracted from tea showed inhibitory activity against SARS-CoV with an $IC_{50}$ of 3 μM[3]. The main protease of coronaviruses is essential for the processing of the replicase complex nonstructural proteins. In addition, the nasal epithelium is critical in the processing of viral spike protein priming for viral infection, and the cell surface protease transmembrane protease serine 2 (TMPRSS2) is highly expressed in the small airway epithelium[4]. Since the outbreak of novel coronavirus, causing severe pneumonia and sequelae in late 2019, drug candidates for SARS-CoV have been re-tested for SARS-CoV-2. Wang et al. reported fruits tannic acids are dual inhibitors targeting viral main protease ($3CL^{pro}$) and host TMPRSS2 with $IC_{50}$ of 13.4 μM and 2.31 μM respectively[5]. However, the potency is weak, and it appears that crude tannic acids cannot reach the therapeutic level without administration in large quantities.

Shih et al. further examined the antiviral activities of proprietary processed tannic acid from Chinese gallnuts, Ultrapure and Potent Tannic Acid (UPPTA). The $EC_{50}$ against SARS-CoV-2 is much improved to 0.5 μM in 3CLpro-overexpressed cellular systems. Moreover, for the first documented human coronavirus infection, HCoV-OC43, the $EC_{50}$ is 1.3 μM. The selectivity index ($EC_{50}/CC_{50}$) declared its effectiveness and safety in vitro. They also evaluated the pharmacokinetics and toxicology of UPPTA to prove its feasibility in drug development[6]. Also, a preliminary report reveals that patients infected by SARS-CoV-2 have a better clinical improvement[7].

As SARS-CoV-2 directly targets the lungs and causes lung damage, treatment of COVID-19 with inhalants may offer more advantages over oral administration. Because the viruses incubate deep in the respiratory tract, the systemically administered drug could not reach them easily. Therefore, high doses of drugs are required through oral or parenteral routes and may cause systemic overexposure and unwanted side effects. The inhaled route of drug administration could provide a much higher concentration in the pulmonary system without much systemic exposure. Moreover, the peripheral metabolism of the drug could be greatly reduced when the drug is directly delivered to the lungs, minimizing systemic drug exposure[8].

Since the toxicity of UPPTA by inhalation has never been investigated, a thorough evaluation is needed before human use and investigational new drug applications. In this study, we evaluated the potential systemic and pulmonary toxicity of UPPTA by inhalation in both Sprague-Dawley (SD) rats and Beagle dogs once a day for 2 consecutive weeks. We also investigated the reversibility of toxicity following a 3-week recovery period after the exposure.

## 2. Materials and methods

The purification and manufacture of UPPTA active pharmaceutical ingredient (API) was described in the previous research[6]; the purity of cGMP-grade APIwas determined to be 98.4%.

### 2.1. Two-week toxicity study in SD rats following a 3-week recovery period

#### 2.1.1. Aerosol generation and analysis

Inhalation exposure system and automatic metered-dose inhaler (MDI) nebulizer system (Beijing Huironghe Technology) were applied. Two bottles of control or test article were triggered to deliver the aerosol at the same time at the frequency of 12 times/min in single-cavity mode. The aerosol flow rate was set at 16 L/min, the dilution flow rate was 0 L/min, and the inner chamber exhaust flow rate is set at 14 L/min.

$T_{99}$ of the exposure system was calculated by 4.6 × V/a, where V is the volume of the chamber (0.00321 $m^3$) and "a" ($m^3$/min) is the total aerosol in flow. During the experiment, the temperature was set at 20–26 ℃, the humidity was set at 30–80%, the oxygen concentration was set at ≥19%, and the carbon dioxide concentration was set at <1%.

For analysis of the concentration of UPPTA, samples were collected with a 3-stage wave plate sampler (5 mL of 30% acetonitrile water was added to each 1-stage wave tube) about 1 min after the beginning of administration. The sampling flow was set at 0.5 L/min. For analysis of the aerosol particle size parameters, including median mass aerodynamic diameter (MMAD), geometric standard deviation (GSD), and fine particle fraction, NGI laryngeal tube (NGI-1342 or NGI-1872, Copley Science, UK) and Copley Inhaler Testing Data Analysis Software, version 3.10 WIBU, were applied. Sample conditions were set at pumping flow rate: 15 L/min; collecting time: 3 min.

### 2.1.2. Animals and treatment

The study procedures were approved by the Institutional Animal Care and Use Committee. 8–9-week-old specific pathogen-free SD rats were purchased from the Zhejiang Vital River Laboratory Animal Technology Co. Ltd., China (license No. SCXK (Zhe) 2019-0001), housed at a maximum of 5 rats per cage and maintained on a 12/12-hour light/dark cycle at 23 ± 4 ℃ and 55 ± 15% humidity. Animals had free access to SPF Rat Growth Breeding Feed diet (Jiangsu Xietong Pharmaceutical Bio-Engineering) and filtered water except during designated procedures. After the initial health examination, all animals were placed in a fixator for about 60 minutes once daily for 5 consecutive days for acclimation. Animals without abnormal behaviors during fixation acclimation were grouped and acclimated for an additional 5 days. All experiments were conducted in JOINN Laboratories (Suzhou) Co., Ltd. in accordance with the Good Laboratory Practice (GLP) guideline.

### 2.1.3. Experimental design

The objectives of this study are to evaluate the toxicity and toxicokinetic properties of UPPTA administered by MDI followed by a 3-week recovery period.

In the toxicity study, a total of 120 rats (60 rats/sex) were randomly assigned into 4 groups (15 animals/sex/group). Animals in Group 1 were given a 60-minute placebo by MDI as an excipient control. Animals in Groups 2 to 4 were given 15-minute, 30-minute, and 60-minute inhalations of UPPTA aerosol by MDI as low, medium, and high dose groups, respectively. The dosing frequency was once daily for 14 consecutive days for a total of 14 doses. The first dosing day was defined as Day 1. The actual delivered dose was calculated with the formula: dose (mg/kg) = ventilation volume per minute (RMV) × concentration × duration/BW/1000, whereas the RMV = 0.608 × BW $(kg)^{0.852}$.

Clinical observation (including daily clinical observation, detailed clinical observation, and local observation), body weight, food consumption, body temperature, ophthalmoscopic examinations, respiratory function (tidal volume, respiratory frequency, and minute respiratory volume), and clinical pathology (hematology, coagulation, clinical chemistry, and urinalysis) were evaluated during the study. Ten animals per sex per group were euthanized at the end of the dosing period (Day 15), and five animals per sex were euthanized at the end of the recovery period (Day 36) for macroscopic examination and complete necropsy examination in each group. Major organs were weighed, and histopathological examination was performed.

In the toxicokinetic study, a total of 56 rats (8 rats/sex of dosing groups; 4 rats/sex of control group) were randomly assigned into 4 groups. Animals in Group 1 were given a placebo by MDI for 60 min as

an excipient control. Animals in Groups 2 to 4 were given 15-minute, 30-minute, and 60-minute UPPTA aerosols by MDI as low, medium, and high dose groups, respectively. The dosing frequency was once daily for 14 consecutive days for a total of 14 doses. The first dosing day was defined as Day 1. The actual delivered dose was calculated with the formula: dose (mg/kg) = ventilation volume per minute (RMV) × concentration × duration/BW/1000, whereas the RMV = 0.608 × BW $(kg)^{0.852}$. Blood samples (approximately 0.3 mL, with EDTA-$K_2$ anticoagulation) were collected via jugular veins from animals of Groups 1 to 4 for toxicokinetic analysis.

### 2.1.4. Clinical observation

All rats were observed twice daily (a.m. and p.m.) during the study for signs of, but not limited to, mortality, moribundity, mental/behavior status, respiration, secretion, feces, and water and food intake.

Detailed clinical observations included, but were not limited to, mental status, behavior, skin, fur, eyes, ears, nose, abdomen, external genitalia, anus, limbs, feet, and respiration were conducted prior to first dosing and once weekly after dosing.

Local observation of irritation at the snout and other contact sites with aerosol and the reaction symptoms (such as swelling, asthma, cough, asphyxia, and other symptoms) of animals after dosing were conducted as well.

### 2.1.5. Clinical pathology

Blood samples for hematology, coagulation, and clinical chemistry determinations were collected via abdominal aorta prior to scheduled euthanasia. Rats were fasted overnight, then blood samples were collected into tubes containing EDTA-K2, sodium citrate, or separation gel and coagulation promoters for hematology, coagulation, or clinical chemistry, respectively. For analysis, Sysmex® XN Hematology System, Sysmex® CS-2000i, Sysmex CS-5100, and TBA-120FR were used for hematology, coagulation, and clinical chemistry analysis, respectively. Urine was collected using metabolism cages and analyzed by the Cobas®6500 automatic urine analyzer.

### 2.1.6. Toxicokinetic study

Blood samples were collected from animals of the control group at the pre-dose time point and 4 h after the end of dosing on Days 1 and 14. Blood samples were collected from animals of the dosing groups at the pre-dose time point, the end of dosing immediately (±2 min), and at post-dosing at 30 min, 1 h, 2 h, 4 h, 8 h, and 24 h on Day 1 and Day 14. After blood collection, the blood samples were mixed by inverting at least 5 times and stored in an ice box. Plasma was separated by centrifugation at 2000 g for 10 min at 4 ℃ within 1 h after blood collection. The plasma was dispensed in duplicate into labeled centrifuge tubes and stored below –60 ℃. The analysis of the biological sample was performed by a validated LC-MS/MS method for the determination of plasma levels.

### 2.1.7. Animal euthanasia and pathology examination

Animals were euthanized with isoflurane inhalation followed by abdominal aorta exsanguination on Day 15 and Day 36. For histopathological evaluation, tissues/organs were fixed in 10% neutral buffered formalin, followed by paraffin sectioning and hematoxylin and eosin (H&E) staining. A five-step grading system (minimal, slight, moderate, marked, or severe) was applied to rate the severity of microscopic lesions.

## 2.2. Two-week toxicity study in Beagle dogs following a 3-week recovery period

### 2.2.1. Aerosol generation and analysis

If not specified, the aerosol and its analysis were the same as those in the rodent study. For the dog experiment, two bottles of control or test article were triggered at the same time at the frequency of 8 times/min in single-cavity mode. The aerosol flow rate was set at 20 L/min, the dilution flow rate was 8 L/min, and the exhaust flow of the inner chamber was set at 26 L/min.

### 2.2.2. Animals and treatment

6 to 7-month-old conventional-grade Beagle dogs were purchased from Jiangsu Marshall Biotechnology (license No. SCXK (Zhe) 2019-0012), housed individually in stainless steel cages, and maintained on an approximate 12/12-hour light/dark cycle at 22 ± 4 ℃ and 55 ± 15% humidity. Animals had free access to a certified canine diet (Beijing Keaoxieli Feed) and tap water. After initial health examination, all animals underwent simulated domestication of inhalation for at least 5 days, about 135 minutes per day. Animals without abnormal behaviors during acclimation were grouped and acclimated for an additional 5 days. The study procedures were approved by the Institutional Animal Care and Use Committee.

### 2.2.3. Experimental design

To evaluate the potential toxicity and toxicokinetic properties, UPPTA was administered by MDI in Beagle dogs once a day for 2 consecutive weeks, and the reversibility of toxicity following a 3-week recovery period was also investigated.

A total of 40 Beagle dogs (20/sex) were randomly assigned into 4 groups (5/sex/group). Animals in Group 1 were given a 90-minute inhalation of placebo by MDI. Animals in Groups 2 to 4 were given 10-minute, 30-minute, and 90-minute UPPTA by MDI at low, medium, and high doses, respectively. Dosing frequency was once daily for 2 consecutive weeks for a total of 14 doses. The day of the first dosing was defined as Day 1.

Placebo group received excipient control. The actual delivered dose was calculated with the same formula as in the rodent study. Animals were evaluated for clinical observation, body weight, food consumption, body temperature, electrocardiogram, blood pressure, ophthalmological examination, and clinical pathology, which are similar to the rodent study. Blood samples were collected on Day 1 and Day 14 for toxicokinetic analysis. At the end of the dosing period, the first 3 animals/sex in Groups 1 to 4 were euthanized 2 weeks after dosing (Day 15). The remaining animals were euthanized at the end of the 3-week recovery period (Day 36). Systematic necropsy was performed for animals in Groups 1 to 4. Also, organ weight, gross anatomy, and histopathology were examined.

### 2.2.4. Clinical observation and clinical pathology

The clinical observation processes are the same as in the rat study. For clinical pathology, the equipment and procedures in the canine study are the same as those used in the rodent study except for the ADVIA 2120 Hematology System, which was used for hematology, and the blood samples were collected via forelimb vein.

### 2.2.5. Toxicokinetic study

Blood samples (approximately 1 mL) were collected via forelimb subcutaneous vein for bioanalysis and toxicokinetic analysis. Blood samples were collected from animals of the control group at the pre-dose time point and at post-dosing 2 h on Day 1 and Day 14. Blood samples were collected from each animal in the dosing groups at the pre-dose time point, after the end of dosing immediately, and at post-

dosing 2, 4, 8, 12, and 23 h on Day 1. On Day 14, blood samples from 3/sex animals of the dosing groups were collected at the pre-dose time point, at the end of dosing immediately, and at post-dosing 2, 4, 8, 12, and 23 h. Blood samples from other 2/sex animals of dosing groups were collected at the pre-dose time point, at the end of dosing immediately, and at post-dosing 2, 4, 8, 12, 23, 48, 72, 96, 120, 144, 264, 384, and 504 h.

#### 2.2.6. Animal euthanasia and pathology examinations

Animals were euthanized after intramuscular injection of Xoletil 50 (12 mg/kg, 50 mg/mL) and xylazine hydrochloride (1 mg/kg, 20 mg/mL), followed by femoral artery bloodletting on Day 15 and Day 36. Histopathological evaluations were the same as in the rodent study.

### 2.3. Test article concentration in lung tissue

UPPTA was administered by MDI in SD rats and Beagle dogs once a day for 1 consecutive week. Frozen rats and dog lung samples were completely thawed in a cooler filled with ice and homogenized well. 20 μL rat lung matrix, 50 μL of internal standard (IS) (200 ng/mL), and 300 μL of tannase reaction solution (1 mg/mL) were added into an Eppendorf tube and mixed well by vortex. The mixtures were incubated at 30 ℃ for 4 h to complete the hydrolysis reaction. The hydrolysate was extracted with 3300 μL of extraction reagent (1.5% (w/w) formic acid in ACN), vortexed for about 2 min and then centrifuged at 12,000 rpm for 10 min at 2–8 ℃. The supernatant was collected and followed by evaporating to dryness under $N_2$. The dried extract was reconstituted in 200 μL reconstitution solution and filtered through a 0.22 μm membrane filter. The analysis of the biological sample was performed by a validated LC-MS/MS method for the determination of tissue levels.

### 2.4. Statistical analysis

Data from male and female animals were analyzed separately. All statistical tests were conducted as 2-sided tests, and the level of significance was set at 0.05 or $p \leq 0.05$. Group means and standard deviations (mean ± SD) were calculated using the Provantis system (SAS 9.2). Data were analyzed with the Levene's test to assess variance homogeneity. Data with homogeneous variance ($p > 0.05$ in Levene's test) were analyzed with the one-way ANOVA followed by the Dunnett's test for multiple comparisons. Data with variance heterogeneity ($p \leq 0.05$ in Levene's test) were logarithmically transformed using the natural logarithm (ln transformation). The Kruskal-Wallis test (non-parametric method) followed by the two-independent-sample test (Mann-Whitney U) for multiple comparisons was performed on the original data when transformed data showed variance heterogeneity.

When a dataset contained negative values, the logarithmical transformation was not applied, and either the original data or the rank-transformed data were used for statistical analysis. When a dataset had zero values, the zero values were regarded as 1/10 of the smallest positive value in the dataset when the logarithmical transformation was performed.

## 3. Results

### 3.1. Analysis findings of the test article

In the SD rodent study, aerosol concentrations were analyzed from Day 1 to 14, and the aerosol particle size distribution was analyzed after the dosing on Days 1, 7, and 14. The actual delivered dose was 2.88 ± 0.96 mg/kg for the low-dose group, 5.35 ± 1.28 mg/kg for the medium-dose group, and 11.70 ± 2.20 mg/kg for the high-dose group. The actual delivered dose and aerosol particle size distribution parameters are presented in **Table 1**.

**Table 1.** The actual delivered dose and aerosol particle size distribution parameters.

| Dose | Low | Medium | High |
|---|---|---|---|
| **SD rat** | | | |
| Target dose (mg/kg) | 3 | 6 | 12 |
| Actual delivered dose (mg/kg) | 2.88 ± 0.96 | 5.35 ± 1.28 | 11.70 ± 2.20 |
| Average MMAD (μm) | 3.11 | 3.07 | 3.26 |
| Average GSD | 1.55 | 1.56 | 1.57 |
| Average FPF (%) | 85.49 | 85.33 | 81.1 |
| $T_{99}$ (min) | 0.92 | | |
| **Beagle dog** | | | |
| Target dose(mg/kg) | 0.3 | 1.0 | 3.0 |
| Actual delivered dose(mg/kg) | 0.28 ± 0.09 | 0.98 ± 0.18 | 3.05 ± 0.48 |
| Average MMAD(μm) | 3.37 | 3.37 | 3.37 |
| Average GSD | 1.49 | 1.49 | 1.49 |
| Average FPF (%) | 82.81% | 82.81% | 82.81% |
| $T_{99}$ (min) | | 0.49 | |

Note: MMAD: Mass Median Aerodynamic Diameter, GSD: Geometric Standard Deviation, FPF: Fine Particle Fraction (Fine Particle Fraction, MMAD < 5 μm). The $T_{99}$ calculation formula of the exposure system is $T_{99} = 4.6 \times V/a$, V is the exposure tower volume (0.00321 $m^3$), $a$ is the set total aerosol generation and dilution flow ($m^3$/min), $a$ = 0.016 $m^3$/min for this test.

In the Beagle dog study, aerosol concentrations were analyzed from Days 1 to 14, and the aerosol particle size distribution was analyzed after the dosing on Days 1, 7, and 14. The actual delivered dose was 0.28 ± 0.09 mg/kg in the low-dose group, 0.98 ± 0.18 mg/kg in the medium-dose group and 3.05 ± 0.48 mg/kg in the high-dose group, respectively. The $T_{99}$ (min) of the exposure system was 0.49 min (T99 = 4.6 × V/a (V = 0.003 $m^3$, a = 0.028 $m^3$/min)). The actual delivered dose and aerosol particle size distribution parameters are in **Table 1**.

## 3.2. Clinical observation and clinical pathology

In the rodent study, there are no test article-related abnormal changes (including daily clinical observation, detailed clinical observation, and local observation), body weight, food consumption, body temperature, ophthalmoscopic examinations, respiratory function, and clinical pathology (hematology, coagulation, clinical chemistry, and urinalysis) in all the dose groups during the study.

In the Beagle dog study, no mortality related to the test article was observed in all three dose groups during the whole study. There are no abnormalities related to the test article, and no toxicologically significant changes were observed in body weight, food consumption, body temperature, electrocardiogram (limb II ECG), blood pressure, ophthalmic examination, coagulation, clinical chemistry, urinalysis, and gross anatomy in all the animals.

## 3.3. Toxicokinetic study

In the SD rodent study, Airnecflu®, UPPTA was not detected after 15 min, 30 min, and 60 min inhalation in most of the plasma samples.

In the Beagle dog study, after the first (Day 1) and last dosing (Day 14), Airnecflu®, UPPTA was not detected in all the plasma samples of the excipient control group. The key TK parameters of Airnecflu® and UPPTA in the low, middle, and high dose groups are shown in **Table 2**.

**Table 2.** The key toxicokinetic parameters of Airnecflu®, UPPTA in the Beagle dog study.

| Analyte | Study day | Dose level (mg/kg) | Gender | $T_{max}$ (hr) | $C_{max}$ (ng/mL) | $AUC_{0-23}$ (hr·ng/mL) |
|---|---|---|---|---|---|---|
| Airnecflu® UPPTA | D1 # | 0.28 | Male | - | - | - |
| | | | Female | - | - | - |
| | | 0.98 | Male | 15.67 ± 6.35 | 103.02 ± 49.40 | 1453.46 ± 719.50 |
| | | | Female | 12.00 ± 0.00 | 81.93 ± 36.90 | 1233.66 ± 739.20 |
| | | 3.05 | Male | 14.20 ± 4.92 | 230.93 ± 98.45 | 3920.88 ± 1593.09 |
| | | | Female | 12.00 ± 0.00 | 356.43 ± 129.60 | 6237.47 ± 1984.03 |
| | D14 # | 0.28 | Male | 0.40 ± 0.89 | 90.10 ± 21.52 | 1773.31 ± 338.18 |
| | | | Female | 0.80 ± 1.79 | 126.14 ± 63.62 | 2785.10 ± 1219.51 |
| | | 0.98 | Male | 2.40 ± 3.57 | 432.62 ± 121.85 | 8621.89 ± 2491.84 |
| | | | Female | 1.61 ± 3.57 | 738.87 ± 475.47 | 15077.32 ± 10791.66 |
| | | 3.05 | Male | 2.01 ± 1.99 | 1162.13 ± 613.63 | 24206.36 ± 12767.67 |
| | | | Female | 7.21 ± 6.56 | 1684.23 ± 841.22 | 34553.42 ± 16559.60 |

-: Data under detectable limits; #, $n = 5$ for each group.

The analysis of the two-sided *t*-test was applied in comparing the sexual difference of toxicokinetic parameters. The result showed that the exposure ($AUC_{0-23}$) of Airnecflu®, UPPTA, had no statistical difference ($p > 0.05$) in both genders after inhalation of Airnecflu®, UPPTA at doses of 0.28, 0.98, and 3.05 mg/kg. The ratios (female/male) of mean $C_{max}$ ranged from 0.80 to 1.71, and the ratios (female/male) of mean $AUC_{0-23}$ ranged from 0.85 to 1.75 on the first dosing day (D1) and after repeated administration (D14). No gender differences were observed in the exposures of Airnecflu®, UPPTA.

In the Beagle dogs, after inhalation of Airnecflu®, UPPTA aerosol at doses of 0.98 and 3.05 mg/kg on Day 1, the mean exposure ($AUC_{0-23}$) in plasma increased dose-dependently in both genders. On Day 14, after inhalation of Airnecflu®, UPPTA aerosol at doses of 0.28, 0.98, and 3.05 mg/kg for 2 consecutive weeks, the mean exposure ($AUC_{0-23}$) increased dose-dependently in both genders. The ratio of mean exposure was slightly greater than the ratio of dose when comparing low dose to medium or high dose.

After consecutive 14-day administration of Airnecflu®, UPPTA at 0.98 and 3.05 mg/kg levels to Beagle dogs, the mean of Airnecflu®, UPPTA $C_{max}$ ratios (Day14/Day1) ranged from 5.53 to 9.84 and the mean of $AUC_{0-23}$ ratios (Day14/Day1) ranged from 6.78 to 13.87 (**Table 2**).

## 3.4. Organ weight change and pathology

In the SD rat study, test article-related increases in the absolute organ weight, organ-to-body weight ratio, and organ-to-brain weight ratio of lung with bronchi were observed in all dosing groups at the terminal necropsy (Day 15) (**Table 3**). These organ weight changes were probably correlated to the following pathology changes.

In the SD rats, test article-related minimal to slight alveolar macrophage aggregation and minimal to moderate perivascular/interstitial inflammatory cell infiltration were noted in the lung with bronchi of both sexes. Minimal alveolar epithelium hyperplasia was noted only in the high-dose group in the lung with bronchi (**Figure 1** and **Table 4**). After a 3-week recovery period (Day 36), there is substantial recovery of the test article-related microscopic findings in the lung with bronchi.

**Table 3.** Weight changes in terminal necropsy and their recover on Day 36 of the SD rats.

| **Sex** | | **Male** | | | **Female** | |
|---|---|---|---|---|---|---|
| **Dose** | Low | Medium | High | Low | Medium | High |
| **Inhalation duration (min)** | 15 | 30 | 60 | 15 | 30 | 60 |
| **Terminal necropsy (Day 15) #** | | | | | | |
| **Lung with bronchi** | | | | | | |
| Organ weight | 1.61 g* | 1.56 g* | 1.69 g* | 1.29 g | 1.32 g* | 1.34 g* |
| Organ-to-Body | 0.46%* | 0.46%* | 0.48%* | 0.52%* | 0.52%* | 0.55%* |
| Organ-to-Brain | 78.47%* | 78.28% | 85.76%* | 67.35% | 67.46% | 71.00% |
| **Recovery necropsy (Day 36) ^** | | | | | | |
| **Lung with bronchi** | | | | | | |
| Organ weight | 1.53 g | 1.59 g | 1.67 g | 1.27 g | 1.23 g | 1.24 g |
| Organ-to-Body | 0.35% | 0.35% | 0.38% | 0.47% | 0.47% | 0.47% |
| Organ-to-Brain | 74.42% | 78.70% | 80.65% | 64.00% | 62.88% | 65.06% |

Data Format: Control data were presented in absolute (actual) values for the control group only; the data for dosed animal were presented as a percentage of change relative to the control group data. *$p \leq 0.05$; #, $n = 10$ for each group; ^, $n = 5$ for each group.

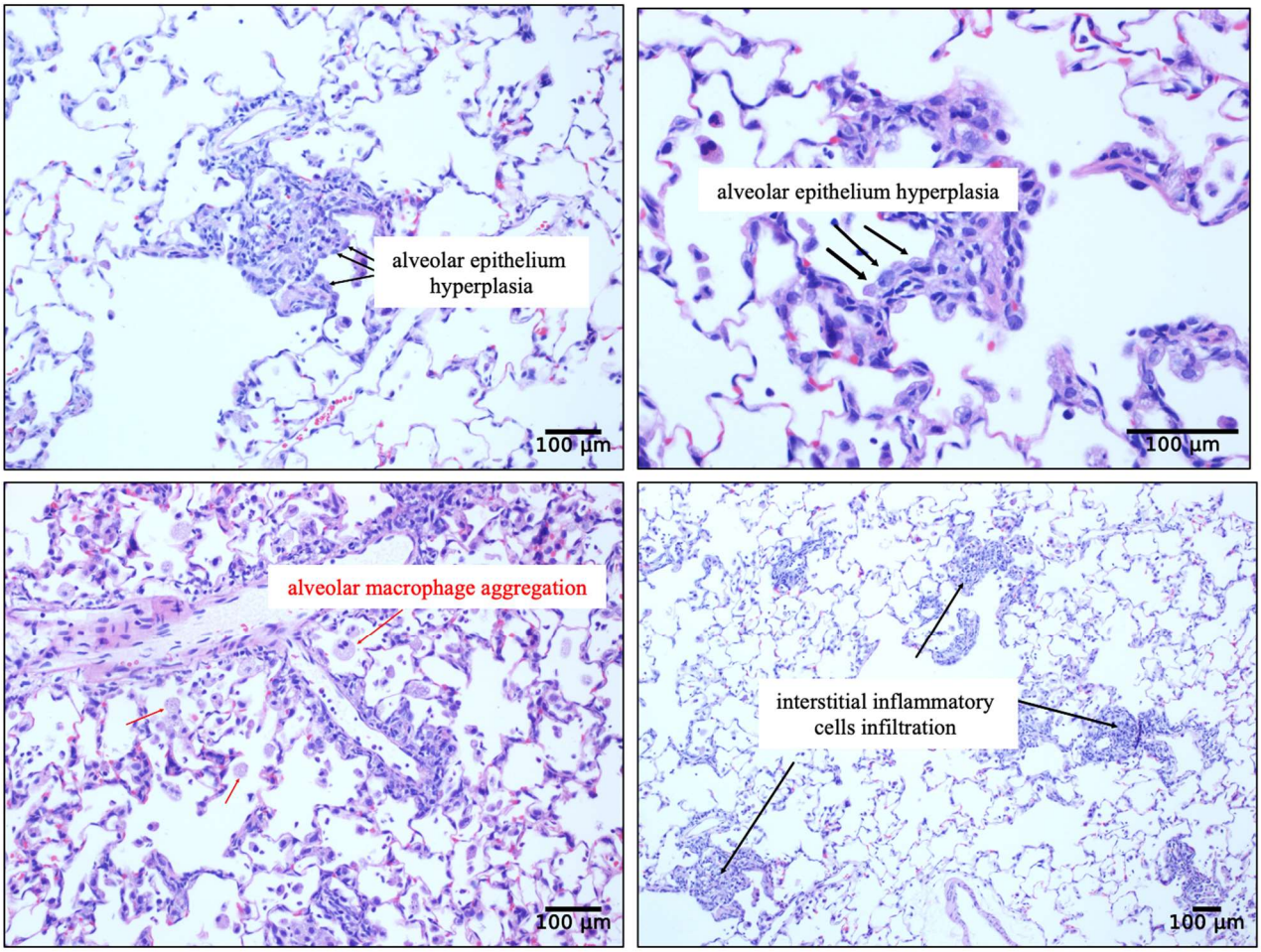


**Figure 1.** Examples of test article-related moderate alveolar macrophage aggregation, moderate interstitial/alveolar inflammatory cells infiltration, and minimal alveolar epithelium hyperplasia were noted in the lung with bronchi in SD rat. Representative lung H&E staining sections of rats with various treatments were displayed. Scale bar = 100 μm.

**Table 4.** UPPTA-related microscopic findings at the terminal and recovery necropsy in SD rat study.

| **Sex** | **Male** | | | | **Female** | | | |
|---|---|---|---|---|---|---|---|---|
| **Dose** | Control | Low | Medium | High | Control | Low | Medium | High |
| **Inhalation duration/time (min)** | 60 | 15 | 30 | 60 | 60 | 15 | 30 | 60 |
| **Terminal necropsy (Day 15) #** | | | | | | | | |
| **Lung with bronchi** | | | | | | | | |
| Alveolar macrophage aggregation | | | | | | | | |
| Minimal | 0 | 8 | 6 | 3 | 0 | 1 | 6 | 3 |
| Slight | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 6 |
| Infiltration, inflammatory cells perivascular; interstitial | | | | | | | | |
| Minimal | 0 | 5 | 3 | 1 | 0 | 10 | 6 | 1 |
| Slight | 0 | 4 | 5 | 2 | 0 | 0 | 4 | 3 |
| Moderate | 0 | 0 | 0 | 7 | 0 | 0 | 0 | 6 |
| Hyperplasia, alveolar epithelium | | | | | | | | |
| Minimal | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 2 |
| **Recovery necropsy (Day 36) *** | | | | | | | | |
| **Lung with bronchi** | | | | | | | | |
| Alveolar macrophage aggregation | | | | | | | | |
| Minimal | 0 | 2 | 0 | 2 | 0 | 0 | 1 | 0 |
| Infiltration, inflammatory cells perivascular; interstitial | | | | | | | | |
| Minimal | 0 | 2 | 2 | 2 | 0 | 0 | 1 | 3 |

#, $n = 10$ for each group; *, $n = 5$ for each group.

In the Beagle dog study, varying degrees of test article-related increases in the absolute weight, organ-to-body weight ratio, and organ-to-brain weight ratio of the lung with bronchi were observed in all dosing groups at the terminal necropsy (Day 15) (**Table 5**). These organ weight changes were probably correlated to the following pathology changes.

**Table 5.** Weight changes in terminal necropsy and their recover on Day 36 of the Beagle dog.

| **Sex** | **Male** | | | **Female** | | |
|---|---|---|---|---|---|---|
| **Dose** | Low | Medium | High | Low | Medium | High |
| **Inhalation duration (min)** | 10 | 30 | 90 | 10 | 30 | 90 |
| **Terminal necropsy (Day 15) #** | | | | | | |
| **Lung with bronchi** | | | | | | |
| Organ weight | 72.12 g | 87.26 g* | 87.69 g* | 64.63 g | 71.08 g | 78.32 g |
| Organ-to-Body | 0.86% | 1.01% | 1.05%* | 0.98% | 1.08% | 1.18% |
| Organ-to-Brain | 100.97% | 118.09% | 122.51% | 90.24% | 106.19% | 112.93% |
| **Recovery necropsy (Day 36) ^** | | | | | | |
| **Lung with bronchi** | | | | | | |
| Organ weight | 73.00 g | 72.50 g | 76.15 g | 68.17 g | 61. 60 g | 79.88 g |
| Organ-to-Body | 0.88% | 0.88% | 0.93% | 0.96% | 0.87% | 1.05% |
| Organ-to-Brain | 105.53% | 93.72% | 97.50% | 96.33% | 90.18% | 111.99% |

Note: Data Format: Control data is presented in absolute terms; the data for dosed animals is presented as a percentage of change relative to the concurrent control group data. *$p \leq 0.05$, #, $n = 3$ for each group; ^, $n = 2$ for each group.

There was test article-related minimal to moderate alveolar macrophage aggregation, minimal to moderate perivascular/interstitial inflammatory cell infiltration, and minimal alveolar epithelium hyperplasia in both sexes' lungs with bronchi. In addition, test article-related minimal to slight increased cellularity of lymphocytes in cortex and minimal inflammatory cells infiltrated in medulla were noted in both sexes' bronchial lymph nodes (**Table 6**). After a 3-week recovery period (Day 36), although test article-related microscopic findings were still observed in the lung with bronchi, the severity and incidence of the above lesions were significantly lower than those observed at the terminal necropsy (Day 15), indicating a substantial recovery.

**Table 6.** UPPTA-related microscopic findings at the terminal and recovery necropsy in the Beagle dog study.

| **Sex** | **Male** | | | | **Female** | | | |
|---|---|---|---|---|---|---|---|---|
| **Dose** | Control | Low | Medium | High | Control | Low | Medium | High |
| **Inhalation duration (min)** | 90 | 10 | 30 | 90 | 90 | 10 | 30 | 90 |
| **Terminal necropsy (Day 15) #** | | | | | | | | |
| **Lung with bronchi** | | | | | | | | |
| Alveolar macrophage aggregation | | | | | | | | |
| Minimal | 0 | 0 | 1 | 0 | 0 | 0 | 2 | 0 |
| Slight | 0 | 0 | 1 | 2 | 0 | 0 | 1 | 2 |
| Moderate | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| Infiltration, Inflammatory cells, interstitium/perivascular/alveous | | | | | | | | |
| Minimal | 1 | 0 | 1 | 0 | 1 | 2 | 1 | 0 |
| Slight | 0 | 0 | 1 | 2 | 0 | 0 | 2 | 2 |
| Moderate | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| Hyperplasia, alveolar epithelium, bronchial epithelium | | | | | | | | |
| Minimal | 0 | 0 | 1 | 3 | 0 | 0 | 0 | 1 |
| **Lymph node, bronchial** | | | | | | | | |
| Increased cellularity, lymphocytic, cortex | | | | | | | | |
| Minimal | 0 | 1 | 0 | 1 | 0 | 0 | 2 | 0 |
| Slight | 0 | 0 | 1 | 2 | 0 | 0 | 1 | 3 |
| Infiltration, inflammatory cells, medulla | | | | | | | | |
| Minimal | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 3 |
| **Recovery necropsy (Day 36) *** | | | | | | | | |
| **Lung with bronchi** | | | | | | | | |
| Infiltration, inflammatory cells, interstitium/perivascular | | | | | | | | |
| Minimal | 0 | 1 | 2 | 0 | 0 | 1 | 0 | 1 |

#, $n$ = 3 for each group; *, $n$ = 2 for each group.

## 3.5. Test article concentration in lung tissues

Airnecflu® UPPTA aerosol was administered to SD rats and Beagle dogs by repeated inhalation at actual mean delivered doses of 26.87 mg/kg and 8.10 mg/kg, respectively, once daily for 7 consecutive days. Test article concentration was measured in lung tissues after animal euthanasia. 425.90 and 10.46 μg UPPTA per gram tissue were detected in 3 rat whole lung tissues and 2 canine accessory lobes of lung, respectively.

## 4. Discussion

Tannic acids have been used in many regions worldwide for multiple therapeutic applications. In Eastern countries, they were applied in traditional treatments in the herbal recipe for various diseases such as diarrhea, bleeding, toxification, dementia, and coughing[1,9,10]. In Western countries, tannic acids were used for toxification, burn injuries, and diarrhea[11–13].

Commercial crude tannic acids are derived from various origins, such as sumac galls, Aleppo oak galls, or sumac leaves. Among these, Chinese gallnut (*Galla chinensis*), which is produced by the Chinese aphid (*Schlechtendalia chinensis)* through stimulation of sumac leaves (*Rhus chinensis*), is the main resource of tannic acids in East Asia for traditional Chinese medicine[14,15].

The coronavirus' lifecycle starts with binding of the envelope spike protein to its receptor, angiotensin-converting enzyme 2 (ACE2). Through member fusion, the RNA genome is released into the host cytosol, where it is translated into the replicase proteins. The polyproteins, pp1a and pp1ab, are cleaved by a virus-encoded protease ($3CL^{pro}$) into individual replicase complex nonstructural proteins. Replication begins in virus-induced double-membrane vesicles derived from the endoplasmic reticulum (ER). The positive-strand genome then serves as a template for full-length negative-strand RNA and sub-genomic RNA (sgRNA). sgRNA translation results in the synthesis of both structural proteins and accessory proteins that are inserted into the ER-Golgi intermediate compartment (ERGIC) for virion assembly. Finally, subsequent positive-sense RNA genomes are incorporated into newly synthesized virions, which are then secreted from the cell membrane[16–18].

Because of the key role of $3CL^{pro}$ in proteolytic processing during coronavirus replication, it has been considered a critical molecular target for the development of anti-coronavirus drugs. In 2005, Chen et al. screened 720 natural compounds for inhibitory activity against SARS-CoV. Tannic acids, extracted from tea, showed the highest inhibitory activity against $3CL^{pro}$ with $IC_{50}$ of 3 μM[3]. In late 2019, a novel coronavirus causing a severe pneumonia outbreak was lately identified and named SARS-CoV-2. Drug candidates once tested for SARS-CoV were re-tested for being potential treatments for SARS-CoV-2.

Wang et al. reported a molecular analysis that demonstrated that tannic acids from fruit formed a thermodynamically stable complex with SARS-CoV-2 $3CL^{pro}$ at a KD of 1.1 μM[5]. They also tested the inhibitory activity of tannic acid against SARS-CoV-2 $3CL^{pro}$ with $IC_{50}$ of 13.4 μM. $3CL^{pro}$ of SARS-CoV and SARS-CoV-2 are 96% identical at the amino acid level, and only one different residue is presented at the binding pocket[19,20]. It is therefore predicted that tannic acids are effective in inhibiting both SARS-CoV-2 activity in addition to SARS-CoV.

Shih et al. further tested the activities of proprietary purified tannic acids from Chinese gallnut, named ultrapure and potent tannic acid (UPPTA), Pentarlandir®[6]. The $EC_{50}$ against two coronaviral strains, SARS-CoV-2 and HCoV-OC43, the earliest-known human coronavirus, are 0.5 μM and 1.3 μM, respectively, as tested in the $3CL^{pro}$-overexpressed cellular system. Even though SARS-CoV-2 shows only 59% identity of $3CL^{pro}$ at the amino acid level with HCoV-OC43 in the binding pocket[20], the inhibitory activities of Pentarlandir® are very close in both strains. It declares the potential of proprietary tannic acids as broad-spectrum pancoronal antivirus drugs to treat other human coronaviruses with similar $3CL^{pro}$ amino acid identities, such as HCoV-OC43.

The UPPTA is a tannic acid derived from Chinese gallnut (*Galla chinensis*) with a high proportion of 5–12 galloyl moieties as compared to other sources of tannic acid from *Quercus infectoria* and *Rhus chinensis*. In fact, the inhibitory activities of tannic acids are positively correlated with the number of

galloyl groups[21]. In addition, the selectivity index ($EC_{50}/CC_{50}$) of the coronaviruses is above 100 when treating UPPTA, which declares its effectiveness and safety. The pharmacokinetic (PK) and toxicology of UPPTA were also investigated by oral administration. In the PK studies, proprietary processed tannic acids were distributable to the pulmonary system without accumulation[6]. A 14-day repeated dose toxicity study in rats demonstrated its safety with a NOAEL (no-observed-adverse-effect level) value above 2000 mg/kg/day[6]. These findings support UPPTA for further drug development for coronaviruses.

Though the safety profile of UPPTA by oral administration has been examined thoroughly, the potential toxicity of Airnecflu® UPPTA by MDI administration has never been investigated. In this study, we evaluated the potential toxicity and the target organ toxicity of Airnecflu® UPPTA by inhalation in both SD rats and Beagle dogs, once a day for 2 consecutive weeks. All the procedures were under GLP regulations.

In our previous studies, we evaluated the acute and 7-day repeated dose toxicity of Airnecflu® UPPTA by inhalation in SD rats and Beagle dogs. The actual mean delivered doses used in the single dose and 7-day repeated dose toxicity of the rat studies were up to 139.20 and 26.87 mg/kg/day, respectively. In the Beagle dog studies, the actual mean delivered doses used in single dose and 7-day repeated dose toxicity were up to 12.40 and 8.10 mg/kg/day, respectively. These doses were defined as maximum tolerated dose (MTD) as there was no death, mortality, and/or significant systemic toxicity observed. Therefore, the expected delivered doses in the 14-day repeatedly toxicological evaluation were set at 12.00 and 3.00 mg/kg/day in SD rat and Beagle dog studies, respectively.

In this study, our findings revealed no death, mortality, or significant systemic toxicity at the actual mean delivered dose of Airnecflu® UPPTA up to 11.70 ± 2.20 mg/kg and 3.05 ± 0.48 mg/kg in SD rats and Beagle dogs, respectively. In the pathology study, test article-related changes, including alveolar macrophage aggregation, perivascular/interstitial inflammatory cell infiltration, and alveolar epithelium hyperplasia, were mostly minimal to slight in SD rats and Beagle dogs. Furthermore, after a 3-week period, substantial recovery of the test article-related microscopic findings was observed in the lung with bronchi in both SD rats and Beagle dogs, indicating a reversible pathology that can recover.

In the toxicokinetic study, Airnecflu® UPPTA was not detected in most of the plasma samples of rats, indicating that it rarely enters the peripheral circulation after inhalation. However, in the canine toxicokinetic study, Airnecflu® UPPTA was detected in the plasma of a Beagle dog at a very low level ($C_{max}$ ~1 μM; molecular weight of UPPTA is 1465 g/mol), and the mean exposure ($AUC_{0\text{-}23}$) increased dose-dependently. The ratio of mean exposure was slightly greater than the ratio of dose when comparing low dose to medium or high dose after 14 days of administration. We also measured the concentration of Airnecflu® UPPTA in lung tissues at the end of the 7-day dosing period from the previous study. Substantial levels of UPPTA were detected in the lung tissues. Taken together, there is a significant gradient of distribution of UPPTA when administered by MDI; UPPTA can be administered successfully by MDI to pulmonary tissue, and there is little UPPTA distributed systemically.

Janower et al. reported potential hepatotoxicity caused by tannic acid through enema administration in a few cases after barium enema examinations[22]. However, liver toxicity was considered to be secondary to tannic acid administration with the performance of barium enemas. Shih et al. conducted the 14-day repeated dose toxicity study by oral route under GLP regulations. They evaluated a full panel of toxicology endpoints and found that the purified tannic acid had no significant abnormalities in all organs, biochemical parameters, or other evaluations[6]. In our phase II human study of UPPTA by oral

administration, no serious adverse event (SAE) was noted, and the adverse events (AE) were minimal and negligible[7].

We have reported the virucidal activity of proprietary UPPTA against SARS-CoV-2. Moreover, we have also investigated the virucidal activity of UPPTA against influenza viruses. UPPTA also showed virucidal activity to inhibit the infection of H1N1 and H3N2 at sub-μM concentrations in vitro. In addition, UPPTA further improved influenza-induced body survival rate, weight loss, and subsequent lung damage in vivo (unpublished findings).

Overall, the administration of UPPTA by either oral administration or MDI inhalation was well tolerated. The minimal pathology observed by MDI administration is, to a large extent, reversible. The no observed adverse effect levels (NOAEL) for MDI administration of UPPTA are 11.70 ± 2.20 mg/kg in SD rats and 3.05 ± 0.48 mg/kg in Beagle dogs, respectively. Airnecflu® UPPTA is considered safe for future use against either influenza or coronavirus infection, as it can be effectively delivered to reach therapeutic concentration in the target organ and minimize systemic exposure.

## Author contributions

Conceptualization, LPL, YWM and GET; methodology, LPL, YWM and GET; software, LPL and YWM; validation, LPL and YWM; formal analysis, LPL and GET; investigation, LPL and TT; resources, TYC and GET; data curation, LPL, YWM and TT; writing—original draft preparation, LPL and YAL; writing—review and editing, LPL, YAL, TT and GET; visualization, LPL and GET; supervision, TYC and GET; project administration, LPL and YWM; funding acquisition, GET. All authors have read and agreed to the published version of the manuscript.

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## Conflict of interest

The authors declare no conflict of interest.

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Review Article

# Exposome analysis in toxicology: A comprehensive review

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**ABSTRACT:** Toxicology has extensively evolved with the study of how external agents impact living organisms. This manuscript examines the exposome, a paradigm representing all environmental exposures a human encounters from conception onward, introducing a holistic approach to understanding these effects on health. First coined by Dr. Christopher Wild in 2005, recent interpretations by Miller and Jones emphasize not only the environmental factors but also behavioral influences, internal biochemical processes, and the implications of the human microbiome. These augmentations underscore the body's dynamic responses and continuous adjustments to external challenges. Traditional toxicology, which primarily focused on singular compounds, often overlooked intricate interplays between multifaceted exposures; the exposome aims to bridge this gap. To analyze the vast spectrum of lifetime exposures, various state-of-the-art techniques are in use, such as untargeted high-resolution mass spectrometry, biobanking, biomonitoring, and diverse omics approaches (metabolomics, adductomics, proteomics, and transcriptomics). These methods empower scientists to uncover unknown environmental risks, offering insights into the complex nexus between external exposures and health outcomes.

***KEYWORDS:*** exposome; metabolomics; environmental monitoring; geospatial analysis; toxicology

## 1. Introduction

Toxicology is the scientific study of how various chemical, physical, and biological agents interact with living organisms and impact their health. Over the years, toxicologists have made significant strides in understanding the risks posed by toxic substances, thanks to advances in analytical techniques, risk assessment methodologies, and a broader perspective on exposure. The exposome is a term coined by Dr. Christopher Wild in 2005 and encompasses the totality of human environmental (non-genetic) exposures from conception onwards, representing a promising paradigm in studying the complex interplay between environmental factors and health outcomes[1]. Within the realm of toxicology, exposome analysis offers an innovative approach to understanding the cumulative impact of diverse environmental exposures on human health. It shifts the focus from individual chemical exposures to a more comprehensive view of environmental factors. This article delves into the significance, techniques, challenges, and potential of exposome analysis in toxicology.

## 2. The rationale for exposome analysis

Miller and Jones have redefined exposome as "the cumulative measure of environmental influences and associated biological responses throughout the lifespan, including exposures from the environment, diet, behavior, and endogenous processes"[2]. There are three primary distinctions between Miller's and Jones' interpretation and that of Dr. Wild. The initial difference revolves around the idea of cumulative biological reactions. This notion encompasses the body's continuous adjustments, both beneficial and detrimental, in response to external stimuli and chemicals. This new definition symbolizes how the body copes with these external challenges.

The next distinction in Miller's and Jones' interpretation is the emphasis on behavior. Behavior is understood in an expansive context, encompassing not only individual intentional actions but also those influenced by familial, communal, and societal structures. This new definition extends beyond mere lifestyle habits. It delves deeper into how individuals dynamically engage with their environment, emphasizing the significance of interpersonal relationships, various interactions, and both physical and emotional stress factors.

Lastly, Miller's and Jones' interpretation integrates the concept of internal processes. The human body is a sophisticated network of biochemical interactions, with myriad processes taking place simultaneously. Processes like glycolysis, oxidative respiration, and the activity of microsomal p450 enzymes, among others, continually produce new molecular entities while decomposing others. Additionally, our internal microbial ecosystem, the microbiome, plays a pivotal role and is included in these "internal processes". Although external exposures remain central to this new definition, it's worth noting that the subsequent damage—be it mutations in DNA, changes in epigenetic patterns, or alterations in proteins—is as vital as the external chemicals causing them. These changes not only indicate the actual impact but might also be more accessible for study, even years after exposure. With the realization that genetic factors alone cannot explain the etiology of many diseases, attention has shifted towards environmental exposures. These exposures range from dietary components, pollutants, drugs, and infectious agents to lifestyle factors[3]. Traditional toxicology has focused on studying single compounds or a group of related compounds, but this approach may miss the complex interactions between multiple exposures. The exposome promises to fill this gap, offering a holistic perspective.

## 3. Techniques in exposome analysis

To capture the vast array of exposures over a lifetime, several advanced analytical techniques are employed:

Untargeted high-resolution mass spectrometry (HRMS): This allows for the identification and quantification of thousands of chemicals in biological samples without prior knowledge of their presence[4].

Biobanking and biomonitoring:

Collections of biological samples (e.g., blood, urine, tissues) stored for future analyses can be crucial for exposome studies. Over time, these samples can be tested for new or previously unmeasurable exposures[5].

Omics techniques:

Metabolomics: Analyses the suite of small molecules (metabolites) in body fluids to infer exposures.

Techniques like NMR spectroscopy and chromatography-mass spectrometry are used to measure metabolite signals, capturing over 10,000 endogenous and exogenous metabolites. By examining metabolites, one can get a sense of the chemicals and processes occurring within the body due to external exposures.

Adductomics: Focuses on identifying adducts, which are products of the reactions of toxicants with DNA or proteins. Identifying adducts can indicate exposure to specific toxins.

Proteomics and transcriptomics: Study of proteins and RNA transcripts to understand changes in cellular processes and pathways upon exposure. High-resolution metabolomics and other untargeted analytical techniques used in exposome studies can identify novel toxicants or biomarkers of exposure without prior hypotheses. This unbiased approach can lead to the discovery of previously unrecognized environmental risks. Proteomics focuses on the study of post-translational modifications to proteins at the cellular level. This approach leverages techniques such as soft ionization mass spectrometry and antibody microarrays. While it offers a breadth of coverage, capturing unknown proteins and protein complexes, its throughput ranges from low to medium. Transcriptomics offers nucleotide-level resolution of RNA expression, a pivotal aspect in understanding gene expression patterns. Hybridization-based technologies, notably RNA-seq, are the cornerstones of this approach. The capacity to identify any sequences that are part of the chosen method or technique makes its coverage expansive. Hybridization-based technologies, in particular, offer high throughput.

In the realm of epigenomics, the focus lies on DNA methylation patterns. Researchers commonly use tools such as the Illumina Methylation EPIC Bead Chip and the 850 K DNA methylation array. The coverage of this approach primarily targets promoters, CpG islands, shores, and open seas. These areas have historically shown variability across different tissues or in various disease states. The throughput for epigenomics stands at a medium to high range. Lastly, high-throughput screening is an innovative approach that measures the activities of a myriad of receptors, including but not limited to estrogen, androgen, and G-protein signaling. Techniques like chemically activated Luciferase gene expression (CALUX) and high-content analysis are at the forefront of this approach. While CALUX can target selected receptors across a vast media range, high-content analysis provides a medium throughput[6].

Environmental monitoring and sensors:

Wearable environmental sensors can track individual exposure to specific environmental factors in real-time, such as UV radiation or air pollution[7,8].

Stationary environmental sensors can provide data on pollutant levels in specific locales, like water quality sensors or air quality monitors[9].

Data Integration and Bioinformatics: Advanced computational tools are crucial for the management, integration, and interpretation of the massive datasets generated in exposure studies[10]. Machine learning and statistical models can be employed to discern patterns, correlations, and potential causations between exposures and health outcomes. The concept of the exposome wheel has been proposed as a way to visualize and organize exposome data[11]. The exposome wheel considers the temporal and spatial dimensions of exposures and their interactions, helping researchers explore the complex web of environmental factors that contribute to an individual's exposome.

Geospatial analysis:

Using tools like Geographic Information Systems (GIS), researchers can map environmental

exposures and correlate them with health outcomes in specific regions or populations. Taking a comprehensive approach to assessing environmental exposure, the EXPOsOMICS project focuses on the "external exposome", or the totality of environmental exposures encountered from birth onwards. Using advanced technologies and large-scale European cohorts, EXPOsOMICS aims to capture a vast array of data on environmental factors, including air and water pollutants, physical activity, and more. By understanding these exposures and their fluctuations, the project hopes to shed light on how the environment shapes health across lifespans[12].

Longitudinal cohort studies:

Following a group of individuals over time can help establish a timeline of exposures and correlate them with health outcomes. These studies are crucial for establishing potential cause-and-effect relationships in the context of the exposome. One such study is PHENOTYPE. The PHENOTYPE (Positive Health Effects of the Natural Outdoor Environment in Typical Populations in Different Regions in Europe) project delves into the potential health benefits of natural outdoor environments, from urban parks to rural green areas. Given the increasing trend of urbanization, understanding the health implications of natural versus urban settings becomes vital. PHENOTYPE assesses how exposure to green spaces affects health, mental well-being, and vital physiological markers. It also considers how personal and socioeconomic factors can mediate these relationships[13].

Questionnaires and diaries:

Personal logs and surveys can provide valuable information on lifestyle, diet, occupational exposures, and other environmental factors.

Cellular and animal models:

Used to test the effects of specific exposures in controlled settings. These can help infer potential mechanisms of toxicity and guide further human studies.

## 4. Challenges in exposome analysis

The vastness and dynamism of the exposure pose several challenges. While the exposome offers a holistic framework for understanding the environmental determinants of health, its vastness and dynamism pose significant challenges in its analysis. Overcoming these challenges necessitates multidisciplinary collaboration, innovative methodological advancements, and substantial investment in research infrastructure.

Temporal variation: Exposures change over time and capturing these variations is complex[14].

Complexity and diversity of exposures:

The exposome encompasses a myriad of exposures, ranging from chemical agents, biological agents, radiation, and psychosocial factors, to name a few. Given this vastness, capturing every single exposure is a herculean task. Each exposure can have numerous sources, pathways, and health outcomes associated with it. This creates a challenge in exposure assessment and in disentangling the combined effects of various exposures on health outcomes[3].

Chemical unknowns: Many compounds detected, especially through untargeted approaches, are unidentified[15].

Dynamic nature of exposures:

The exposome is not static; it evolves throughout an individual's lifespan, influenced by factors

such as lifestyle changes, relocation, dietary shifts, and more[16]. This temporal variation requires high-frequency longitudinal data collection to capture the nuances of exposure dynamics, which is resource-intensive.

Data volume: The sheer volume of data requires advanced storage, processing, and analytical strategies[17]. The assessment of the exposome generates vast amounts of data. Managing, storing, and analyzing such vast datasets, especially in a manner that ensures data integrity, security, and privacy, is a significant hurdle[18]. Advanced computational tools and infrastructures are required, often necessitating interdisciplinary collaboration between epidemiologists, bioinformaticians, and data scientists.

Lack of standardized measurement tools:

There's an inherent challenge in the absence of universally accepted tools or protocols for measuring all components of the exposome. While advancements like high-resolution metabolomics provide opportunities for untargeted exposure assessment[19], there's a need for standardization to allow comparison and meta-analysis across studies.

Causality: Linking exposures to health outcomes is challenging due to the multifactorial nature of diseases[20].

Interpretability and validation:

Identifying potential biomarkers or molecular signatures from exposome data is only one step. Validating these findings and interpreting them in the context of health outcomes requires rigorous methodological approaches and often experimental validation in model systems.

Integration with genomics:

The ultimate goal is to understand how the exposome interacts with the genome in influence health outcomes. Integrative analyses that consider both genetic and environmental factors are computationally complex and require sophisticated statistical methodologies[21]. By integrating exposome with genomics, researchers can uncover how genetic susceptibilities modulate the body's response to environmental toxicants. Such interactions can explain the variability in toxic responses among individuals[10].

## 5. Applications in toxicology

Several studies have employed exposome analysis to understand disease mechanisms and identify toxic agents. Examples include investigating the role of combined air pollutants in respiratory diseases, unraveling the interplay between pesticides and dietary components in neurodegenerative diseases, and identifying previously unknown environmental risk factors for rare cancers[22]. The future of exposome analysis in toxicology is bright. The evolving technologies and increasing interest in the exposome promise a transformative impact on toxicology through regulatory decisions. As our understanding grows, regulatory bodies might begin to account for combined exposures when setting safety limits. Personal exposure profiles might be used to provide individualized risk assessments for diseases related to environmental exposures[23]. There is a place for therapeutic interventions. Identifying key toxic exposures can lead to personalized intervention strategies to reduce disease risk. With a comprehensive understanding of environmental exposures and their health effects, public health policies can be better tailored. Identification of high-risk exposure combinations or vulnerable subpopulations can guide targeted interventions.

Exposome data, combined with advanced in vitro and in vivo toxicological studies, can shed light

on the mechanistic pathways by which exposures lead to adverse health outcomes. This deepens the understanding of pathophysiology and can inform therapeutic strategies[24]. The dynamic nature of the exposome, evolving with age and life events, allows toxicologists to study the effects of exposures at various life stages, from in-utero to old age. This can reveal critical windows of susceptibility and the long-term health consequences of early-life exposures[25]. Exposome analysis provides a more comprehensive picture of an individual's or population's exposure history, improving the precision of risk assessments. By capturing both peak and cumulative exposures, it can better inform dose-response relationships and vulnerability assessments[26].

## 6. Discussion

By embracing the exposome, toxicology can evolve from a discipline of singular interactions to a more integrated science that considers the full tapestry of life's exposures. This paradigm shift has the potential to transform not just toxicology but also the broader realm of public health, environmental regulation, and personal medicine. Toxicology, at its core, is the study of how external agents affect living organisms. The exposome enriches this understanding by considering the entire spectrum of exposures throughout an individual's life. The biological mechanisms that mediate the effects of these exposures present an orchestra of metabolic and microbiome activities that translate environmental cues into biological outcomes. This approach is akin to looking behind the scenes of a performance, understanding not just the music but also the musicians and instruments at play.

Toxicological research and policy need to integrate the exposure-biology-disease nexus more explicitly. By doing so, we can better predict and mitigate the health impacts of environmental exposures. A more nuanced understanding of this nexus also opens the door to personalized interventions and more targeted public health strategies. Ultimately, the goal is to not only map the exposome but also to harness its insights for more effective prevention and treatment of diseases linked to environmental factors. The nexus between exposure and disease is a complex journey from an external event to an internal biological response, culminating in health outcomes. The exposome offers a lens to view this journey in its entirety, identifying not only the toxic agents but also the biological mediations that lead to disease. It is in this biological mediation that the potential for intervention lies—targeting not just the exposures but also enhancing biological resilience.

The complexity of the exposome cannot be unraveled by toxicology alone. It requires the concerted efforts of epidemiologists, geneticists, biostatisticians, and many others. In this collaborative future, the insights gleaned from exposome research can inform public policy, leading to more effective strategies for disease prevention and environmental health.

## 7. Conclusion

In conclusion, the concept of the exposome represents a significant shift in the field of toxicology, offering a more comprehensive and holistic view of environmental exposures and their impact on health. Exposome analysis, with its multi-omics approaches and integration of diverse data sources, has the potential to revolutionize risk assessment and regulatory decision-making. As our understanding of the exposome continues to evolve, it is essential for researchers, policymakers, and public health professionals to collaborate in order to address the complex challenges and uncertainties associated with exposome-based toxicology. While challenges remain, the potential benefits for public health are enormous, pushing the boundaries of traditional toxicology into a new era.

## Conflict of interest

The authors declare no conflict of interest.

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Review Article

# A review on water pollution by γHCH (lindane) and its removal using nanomaterials

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**ABSTRACT:** Water pollution by the direct discharge of pollutants (fertilizers, pesticides, heavy metals, etc.) into the river without any pretreatment has become a severe environmental/health hazard. Organochlorine pesticides have extensively been used from the 1940s to 1980 as insecticides in agriculture, weedicides, herbicides, etc. Lindane, an organochlorine pesticide, contributes to bioaccumulation in aquatic organisms and biomagnification in the food chain due to its hydrophobic chemical nature and environmentally persistent property with a very slow rate of degradation. Nanotechnology has proven to be very efficient in removing pollutants. Nanomaterials with unique physical and chemical properties have become a tool for toxicant eradication. Some of the properties of nanomaterials, like high reactivity, adhesion, reflectance, surface plasmon resonance to detect toxic materials, quantum effect in which there is no resistance faced by charged particles, small size, and large surface area to volume, enable them to adsorb many toxicants on their surface, thereby assisting in detoxification and removal of pollutants from water. Some examples include the application of nano-zerovalent iron in the oxidation of groundwater, the reusability of photocatalytic membranes, and many more. This review article presents an updated account of some techniques for eradicating lindane from the aqueous medium.

***KEYWORDS:*** lindane; nano catalyst; adsorbents; nanofiltration; nanotubes

## 1. Introduction

The increased outbursts in population, industrialization, and decreased employment ratio have led to the adaptation of urban life technologies. These technologies, on the one hand, are blossoming for the users, but on the other hand, they are making them diseased in the long run. The fantasy of these technologies is that they never leave users barehanded. In the real sense, everybody is living under the shield of technology.

Water pollution has been a very intriguing subject in all fields of science. The water quality of a river is defined by its physicochemical properties like salt, BOD, pH, suspended solids, speed, colligative (concentration, molarity, molality), and non-colligative (viscosity, solubility, surface tension), which play an important role in the growth of plants and animals. In this review article, a comprehensive study has been made on how nanocatalysts, nanofiltration, and nanoadsorbents can effectively reduce the effects of lindane.

## 2. Water pollution by organochlorine pesticides, especially lindane

The γ isomer of hexachlorocyclohexane (HCH) is the most polar and reactive form of all its isomers, α, β, δ, γ, and ε. It is a white crystalline solid with a slight musty odour, melting point 112.5 °C, boiling point 323 °C, vapor pressure 9.4 × $10^{-6}$ mmHg at 20 °C, density 1.85 g/$cm^3$, relative density 1.87, solubility in water (nil), solubility in polar organic solvents 70%–100%, corrosive to aluminum, and non-flammable. Upon heating, it can form toxic fumes of gases such as phosgene, hydrogen chloride, and carbon monoxide. It is slowly metabolized through four possible routes. (i) Dehydrogenation results in gamma-hexachlorocyclohexane; (ii) dehydrochlorination in gamma-pentachlorocyclohexene; (iii) dechlorination to gamma-tetrachlorohexene; and (iv) hydroxylation results in hexachlorocyclohexanol[1–4]. Its use has been banned since 2009 under the Stockholm Convention on persistent organochlorine pesticides[5,6]. Due to their long half-lives, these chemicals persist in the environment for a long time, adversely affecting the quality of the natural ecosystem[7]. The main cause of environmental contamination by lindane is its use in agricultural practices and the intake of foods from treated agricultural commodities. Approximately 12%–30% of lindane volatilizes into the atmosphere, which can again be deposited by rainfall or coming into contact with water bodies (https://en.wikipedia.org/wiki/Lindane#Pest_repellent). So, this cycle is always continued and is known as the grasshopper effect. As a pharmaceutical, it is used for the treatment of lice and scabies; in households as an insecticide; in agriculture as a weedicide or as a fungicide; to treat live stocks, pets, and food crops. Further, their non-hydrophilic and non-biodegradable nature contributes to biomagnification and bioaccumulation in aquatic organisms[8]. As an insecticide, it protects crops from insects like locusts, which have put the economy of farmers at risk. The use of fertilizers and pesticides by farmers to grow crops at a faster rate than their growth at optimal conditions results in increased crop turnover. However, this practice has enormously disrupted the natural flora and fauna of soil and water. About 45% of the crops have been destroyed because of pesticide use[9]. The nanoparticles of fertilizers, pesticides, etc. endanger the environment. It is difficult to predict when its drift in the air will subside as it is extremely stable in the air[10]. **Figure 1** shows the yearly worldwide use of different pesticide formulations[11].

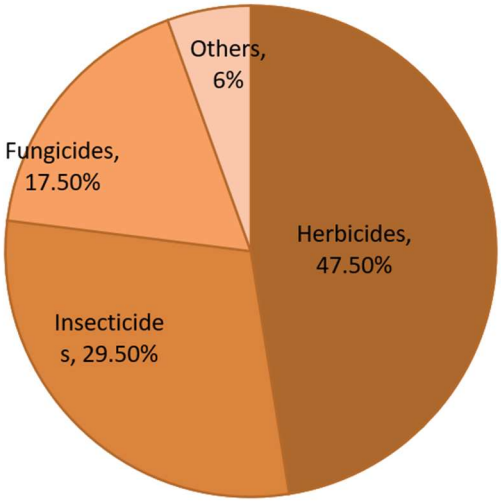


**Figure1.** Worldwide annual pesticide usage.

The human population consuming the fish from any pesticide-contaminated river or pond may be at risk. The residues of lindane have been analyzed in different components like soil, water, air, plants, and even humans. The rhizospheric mycobacterium species p27 can degrade the lindane in soil and help

increase plant growth[12]. The safe drinking water directives determine a limit of 0.5 μg/L for all pesticides in water and 0.1 μg/L for individual pesticides[13]. Acute health effects from lindane exposure or breathing can cause irritation to the skin, eyes, nose, throat, and lungs. It can also cause headaches, nausea, vomiting, dizziness, seizures, irritability, restlessness, muscle weakness, twitching, convulsions, and a coma. Chronic effects can cause cancer, decreased fertility in females, damage to the developing fetus, blood cells causing anemia, kidney damage, liver damage, and arrhythmia (https://nj.gov/health/eoh/rtkweb/documents/fs/1117.pdf). There are numerous studies on lindane toxicity, sublethal exposure of lindane to fish, and changes in enzyme activities that prove its adverse effects on aquatic bodies[14–17]. There are several methods for lindane removal from the water, like the non-thermal plasma method, the use of organ zeolites, and the use of microorganisms (gram-negative) as bioremediation. Among all these methods mentioned and many more, no doubt nanotechnology has proved to be very efficient in lindane removal and wastewater treatment.

## 3. Nanotechnology as a tool to eradicate pollutants

The use of nanomaterials has proven to mitigate the levels of pollutants, including pesticides, which can be dangerous, life-threatening, and pose serious threats to aquatic life. The nanoparticles with large surface area, high reactivity for contaminants, magnetic properties, and surface modifiability make them efficient at removing pollutants from water[18,19]. They show a quantum effect by decreasing the activation energy for the reaction to occur and diffusing into a contamination zone that microparticles cannot access. Engineered nanomaterials like nano-catalysts capture freely available solar energy to decontaminate water under visible light[20]. Nano-metal oxides of titanium and zinc have been used as photocatalysts and adsorbents. Zeolites have been shown to remove heavy metals.

**Nanofiltration** is a technique to remove organic matter and divalent ions from surfaces and groundwater, making it potable and soft. In this process, a membrane (dense/porous) is used that separates solid matter from the aqueous phase. Nanostructured catalytic membranes made of cellulose acetate, polyvinylidene fluoride, polysulphone, etc. are efficient at removing chlorinated toxicants. Properties like molecular weight, membrane pore size, dipole moment, and lipophilicity affect the adsorption of pesticides on membranes. The nanofiltration technique is superior to ultra (0.01 μ) and micro (0.1 μ) filtration, which removes particles in the range of 0.00–0.0001 μ[21,22]. This technique works on a diffusion-permeation mechanism. The uncharged pollutants are separated based on the size exclusion limit of the membrane. In contrast, charged contaminants separate based on charge repulsion/electrostatic interaction and require less pressure (7–30 atm) than reverse osmosis (20–100 atm)[23–27]. Due to the fixed surface charge of nanomembranes, they possess selective binding specificity to various contaminants in the liquid, apart from physical separation. The 90% retention of toxicants on the membrane depends on the membrane's pore size[28]. Hydrogen bonding between organochlorine pesticides and hydrophilic groups on membranes enhances the adsorption of pesticides. The pesticides' polarity/dipole moment helps to retain the toxicants on the membrane; conversely, increased dipole moment leads to lower retention[29]. The formation of macromolecular complexes or natural organic matter also intensifies retention[30]. Adsorption of toxicants increases with increasing ionic strength and decreasing the pH of feed water[31].

Pesticide adsorption on membranes follows pseudo-second-order kinetics due to oxides of metals such as ferric, manganese, aluminum, titanium, and magnesium. The adsorption process is spontaneous and exothermic. It destroys toxicants, converting them into safer byproducts at different temperatures[32]. Oxides of ferric and zinc nanoparticles are potentially useful for the remediation of lindane-polluted

sites because they can reach or penetrate zones that are inaccessible to micro-size solid catalysts[33].

Carbon nanotubes made of graphite and graphene (carbon nanomaterials) have good potential to remove pesticides from water. The small pore size of these tubes allows water to pass at a fast speed while retaining the toxicants[34,35]. The adsorption of organic chemicals on carbon nanotubes (multi/single/hybrid) is due to interactions like the hydrophobic effect, covalent bonding, π-π interactions, hydrogen bonding, and electrostatic interactions, which make them more hydrophilic and suitable for the sorption of low molecular weight and polar compounds. Graphene can adsorb pesticides ranging from 600–2000 mg/g from water[36]. The presence of oxidizing agents tends to increase toxicant retention.

**Nano-catalyst:** Photocatalytic oxidation is a process to degrade non-biodegradable and toxic pollutants into harmless products, carbon dioxide, and water. In this technique, a positively charged semiconductor acts as a catalyst, which absorbs light and generates superoxide/hydroxyll radicals in water, which helps in the oxidative reduction of pollutants[37]. The different types of semiconductor materials used as photocatalysts are ZnO (zinc oxide), $TiO_2$ (titanium dioxide), $Fe_2O_3$ (ferric oxide), CdS (cadmium sulfide), and $WO_3$ (tungsten oxide)[38]. $WO_2$ showed increased adsorption capacity for organic dyes in water, $TiO_2$ for pesticide degradation[39,40], and non-metallic silica oxide for the removal of organic compounds and heavy metals[41,42]. Doping of $TiO_2$ with nitrogen in a 16:1 M ratio showed 100% photocatalytic oxidation of lindane in visible light, whereas 37.5% under UV light[43]. Biopolymers of iron sulfide nanoparticles degraded 94% lindane in 8 h, and 100% degradation of lindane took place under $Fe_3$ (nano)/$H_2O_2$/UV in 320 min of UV irradiation time[44,45]. C18-embedded $Fe_3O_4$ are magnetic nanoparticles used for separating non-polar (organochlorines) and polar pesticides (organophosphates). The magnetic nano-iron particles with enzymes decrease their activation energy and increase longevity, durability, and stability by keeping the catalyst in a reduced state and not letting it oxidize[46]. The studies on biocatalytic dechlorination of lindane have been done using nanoparticles of Pd (0) coated on *Shewanella oneidensis* and Pd/FeO bimetallic nanoparticles with *Sphingomonas* sp. NM05.

**Nanosorbents:** This is a surface phenomenon. The sorption of pollutants on the sorbent surface depends on the transport of contaminants from water to the sorbent surface, adsorption at the sorbent surface, and lastly, transportation within the sorbent. Magnetic nanoadsorbents with a particular ligand affinity bind to organic contaminants as receptors[47]. Iron oxide nanostructures remove organic pollutants and heavy metals from water and can later be regenerated using magnetic separation or catalytic combustion at 300 °C. Granulous activated carbon has been the best for the removal of organic pollutants as it is a thermally and chemically stable material. The nano-zero-valent ions combined with the reactive nanoscale iron products are best for the dechlorination of organochlorine pesticides. They degrade pollutants effectively with minimal generation of secondary pollutants. The nano-zero-valent iron provides complete reductive degradation of lindane into benzene and chloride within 24 h[48]. Some of the biosorbents made from salmon milt DNA hydrogel beads have been used for dioxin eradication[49].

## 4. Conclusion

The unique properties of nanomaterials, like their small size, high reactivity, and eco-friendliness, make them highly efficient for removing toxicants from water. Several studies employing green methods have been done for the eradication of pesticides from the water. Each technique has its own importance, uniqueness, and specificity. Nanozinc oxide with hydrogen peroxide, photo Fenton nanomaterials used as catalysts and adsorption, and many other techniques have been found to be efficient in lindane removal. Though they have proved to be good, their use at the commercial level is also very challenging. Long-term study assessments need to be done to know about different types of risk factors that may arise when

using these technologies.

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## Conflict of interest

The authors declare no conflict of interest.

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# Appendix

**Table A1.** A list of nano technological and bio sorbents to remove pollutants especially lindane.

| Author(s) | Year | Title |
|---|---|---|
| Ningthoujam, et al. | 2023 | Green production of zero-valent iron nanoparticles using pomegranate peel extracts and its use in lindane degradation |
| Kajitvichyanukul et al. | 2022 | Challenges and effectiveness of nanotechnology-based photocatalysis for pesticides-contaminated water: A review |
| Yadav and Ahmaruzzaman | 2021 | Recent advances in the development of nanocomposites for effective removal of pesticides from aqueous stream |
| Uba and Baba | 2021 | The use of plants, nanotechnology and surfactants in lindane remediation |
| Nguyen et al. | 2020 | Removal of lindane from aqueous solution using aluminum hydroxide nanoparticles with surface modification by anionic surfactant |
| Fiorenza et al. | 2019 | Selective photodegradation of 2,4-D pesticide from water by molecularly imprinted $TiO_2$ |
| Taghizade et al. | 2018 | Application of nanotechnology in pesticides removal from aqueous solutions |
| Golshan et al. | 2018 | Photocatalytic activation of peroxymonosulfate by $TiO_2$ anchored on cupper ferrite ($TiO_2$@$CuFe_2O_4$) into 2,4-D degradation |
| Rani et al. | 2017 | Recent strategies for removal and degradation of persistent & toxic organochlorine pesticides using nanoparticles |
| Maddah et al. | 2017 | $Fe_3O_4$/CNT magnetic nanocomposites as adsorbents to remove organophosphorus pesticides from environmental water |
| Khan et al. | 2016 | Efficient degradation of lindane in aqueous solution by iron (II) and/or UV activated peroxymonosulfate |
| Baruah et al. | 2016 | Perspectives and applications of nanotechnology in water treatment |
| El-Kady et al. | 2016 | Evaluation of sorption of lindane on activated carbon derived from rice straw and fungal biomass of phanerochaete chrysosporium |
| Baruah et al. | 2015 | Nanotechnology in water treatment |
| Tanujjal et al. | 2014 | Applications of nanotechnology in wastewater treatment |
| Rizwan et al. | 2014 | Ecofriendly application of nanomaterials: Nanobioremediation |
| Kaur et al. | 2014 | Synthesis and surface engineering of magnetic nanoparticles for environmental cleanup and pesticide residue analysis |
| Jaseetha et al. | 2014 | Degradation of lindane by a novel embedded bio-nano hybrid system in aqueous environment |
| Derbalah et al. | 2014 | Monitoring and remediation of organochlorine residues in water |
| De et al. | 2014 | Worldwide pesticide use. In: *Targeted Delivery of Pesticides Using Biodegradable Polymeric Nanoparticles* |
| Zhang et al. | 2013 | Superior adsorption capacity of hierarchical iron oxide @ magnesium silicate magnetic nano rods for fast removal of organic pollutants from aqueous solution |
| Qu et al. | 2013 | Applications of nanotechnology in water and wastewater treatment |
| Singh et al. | 2013 | An integrated (nano-bio) technique for degradation of γ-HCH contaminated soil |
| Reddy et al. | 2013 | Emerging green chemical technologies for the conversion of $CH_4$ to value-added products |
| Prachi et al. | 2013 | Nanotechnology in waste water treatment |
| Musbah et al. | 2013 | Retention of pesticides and metabolites by nano filtration by effects of size and dipole moment |
| Danwittayakul et al. | 2013 | Enhancement of photocatalytic degradation of methyl orange by supported Zinc Oxide nanorods/Zinc Stannate (ZnO/ZTO) on porous substrates |

**Table A1.** (*Continued*).

| Author(s) | Year | Title |
|---|---|---|
| Coronado et al. | 2013 | Design of advanced photocatalytic materials for energy and environmental applications |
| Bhattacharya et al. | 2013 | Role of nanotechnology in water treatment and purification: Potential applications and implications |
| Behnam et al. | 2013 | Destructive adsorption of diazinon pesticide by activated carbon nanofibers containing $Al_2O_3$ and MgO nanoparticles |
| Palakas et al. | 2012 | Removal of pesticides from water by N.F. and R.O. membranes—A review |
| Fryxell | 2012 | Environmental applications of nano materials |
| El-Safty | 2012 | Topical developments of nanoporous membrane filters for Ultrafine Noble metal nanoparticles |
| Anbia et al. | 2012 | Removal of acid dyes from aqueous media by adsorption onto amino-functionalized nanoporous silica SBA-3 |
| Alvarez et al. | 2012 | Maize plants (Zea mays) root exudates enhance lindane removal by native Streptomyces strains |
| Wu et al. | 2011 | Surface plasmon resonance-induced visible light photocatalytic reduction of graphene oxide: Using Ag nanoparticles as a plasmonic |
| Sarkar et al. | 2011 | Photoselective excited state dynamics in ZnO-Au nanocomposites and their implications in photocatalysis and dye-sensitized solar cells |
| Qiu et al. | 2011 | Controllable corrugation of chemically converted graphene sheets in water and potential application for nanofiltration |
| Kochuveedu et al. | 2011 | Surface-plasmon-induced visible light photocatalytic activity |
| Chan et al. | 2011 | Recent developments of metal oxide semiconductors as photocatalysts in advanced oxidation processes (AOPs) for treatment of dye waste-water |
| Cecen et al. | 2011 | Activated carbon for water and wastewater treatment: Integration of adsorption and biological treatment |
| Cahill et al. | 2011 | Semi-automated liquid chromatographye-mass spectrometry (LC-MS/MS) method for basic pesticides in wastewater effluents |
| Zhang et al. | 2010 | Preparation of carbon coated $Fe_3O_4$ nanoparticles and their application for solid-phase extraction of polycyclic aromatic hydrocarbons from environmental water samples |
| Senthilnathan et al. | 2010 | Photocatalytic degradation of lindane under UV and visible light using N-doped $TiO_2$ |
| Kowalska et al. | 2010 | Visible-light-induced photocatalysis through surface plasmon excitation of gold on titania surfaces |
| Ji et al. | 2010 | First principles calculations of N:H co-doping effect on energy gap narrowing of ZnO |
| Jeon et al. | 2010 | Morphology-controlled synthesis of highly adsorptive tungsten oxide nanostructures and their application to water treatment |
| Hu et al. | 2010 | Synthesis of mono disperse $Fe_3O_4$@silica core-shell microspheres and their application for removal of heavy metal ions from water |
| Fu et al. | 2010 | Focus on the morphology-dependent nano catalysis papers |
| Cloete et al. | 2010 | Nanotechnology in water treatment applications |
| Baruah et al. | 2010 | Enhanced visible light photocatalysis through fast crystallization of zinc oxide nanorods |
| Rehman et al. | 2009 | Strategies of making $TiO_2$ and ZnO visible light active |
| Elliott et al. | 2009 | Degradation of lindane by zero-valent iron nanoparticles |
| Baruah et al. | 2009 | Photoreactivity of ZnO nanoparticles in visible light: Effect of surface states on electron transfer reaction |
| Baruah et al. | 2009 | Nanoparticle applications for environmental control and remediation |
| Ullah et al. | 2008 | Photocatalytic degradation of organic dyes with manganese-doped ZnO nanoparticles |
| Tao et al. | 2008 | Synthesis and optical properties of halogen-doped ZnO phosphor |

**Table A1.** (*Continued*).

| Author(s) | Year | Title |
|---|---|---|
| Satapanajaru et al. | 2008 | Remediation of atrazine-contaminated soil and water by nano zero-valent iron |
| Mauter et al. | 2008 | Environmental applications of carbon-based nanomaterials |
| Graciani et al. | 2008 | Au ↔ N synergy and N-doping of metal oxide-based photocatalysts |
| Drewes et al. | 2008 | Comparing nanofiltration and reverse osmosis for treating recycled water |
| Baruah et al. | 2008 | Visible light photocatalysis by tailoring crystal defects in zinc oxide nanostructures |
| Mertens et al. | 2007 | Biocatalytic dechlorination of lindane by nano-scale particles of Pd(0) deposited on *Shewanella oneidensis* |
| Li et al. | 2007 | Preparation of silica-supported porous sorbent for heavy metal ions removal in wastewater treatment by organic-inorganic hybridization combined with sucrose and polyethylene glycol imprinting |
| Kanade et al. | 2007 | Self-assembled aligned Cu doped ZnO nanoparticles for photocatalytic hydrogen production under visible light irradiation |
| Zhang et al. | 2006 | Self-assembled 3D flowerlike iron oxide nanostructures and their application in water treatment |
| Riu et al. | 2006 | Nanosensors in environmental analysis |
| Liu et al. | 2006 | Nanoparticles and their biological and environmental applications |
| Gorria et al. | 2006 | Synthesis of magnetically separable adsorbents through the incorporation of protected nickel nanoparticles in an activated carbon |
| Adak et al. | 2006 | Fixed bed column study for the removal of crystal violet (C. I. Basic Violet 3) dye from aquatic environment by surfactant-modified alumina. Dyes and Pigments |
| Zhao et al. | 2005 | Adsorption properties of mesoporous silicas for organic pollutants in water |
| Peng et al. | 2005 | Carbon nanotubes-iron oxides magnetic composites as adsorbent for removal of Pb (II) and Cu (II) from water |
| Paknikar et al. | 2005 | Degradation of lindane from aqueous solutions using iron sulfide nanoparticles stabilized by biopolymers |
| Nurmi et al. | 2005 | Characterization and properties of metallic iron nanoparticle: Spectroscopy, electrochemistry, and kinetics |
| Liu et al. | 2005 | Preparation and characterization of DNA hydrogel bead as selective adsorbent of dioxins |
| Fitzgerald et al. | 2005 | Cobalt-doped ZnO—A room temperature dilute magnetic semiconductor |
| Aksu et al. | 2005 | Application of biosorption for the removal of organic pollutants: A review |
| Nghiem et al. | 2004 | Trace contaminant removal with nanofiltration. In: *Nanofiltration—Principles and Applications* |
| Fujihara et al. | 2004 | Tunable visible photoluminescence from ZnO thin films through Mg-doping and annealing |
| Zhang | 2003 | Nano-scale iron particles for environmental remediation: An overview |
| Chaudhary et al. | 2003 | Granular activated carbon (GAC) adsorption in tertiary wastewater treatment |
| Oliveira | 2002 | Activated carbon/iron oxide magnetic composites for the adsorption of contaminants in water |
| Košutić and Kunst | 2002 | Removal of organics from aqueous solutions by commercial RO and NF membranes of characterized porosities |
| Gupta et al. | 2002 | Removal of lindane and malathion from wastewater using bagasse fly ash—A sugar industry waste |
| Bhatkhande et al. | 2001 | Photocatalytic degradation for environmental applications |
| Young et al. | 1998 | The removal of lindane from aqueous solution using a fungal biosorbent |
| Sayles et al. | 1997 | DDT, DDD, and DDE dechlorination by zero-valent iron |

**Table A1.** (*Continued*).

| Author(s) | Year | Title |
|---|---|---|
| Ju et al. | 1997 | Study on the biosorption of lindane |
| Šafařík et al. | 1997 | Adsorption of water-soluble organic dyes on magnetic charcoal |
| Bowen et al. | 1996 | Nanofiltration: Principles and applications |
| Hagfeldt et al. | 1995 | Light-induced redox reactions in nanocrystalline systems |
| Mills et al. | 1993 | Water purification by semiconductor photocatalysis lindane |
| Cadotte et al. | 1988 | Nanofiltration membranes broaden the use of membrane separation technology |

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Review Article

# Immunology, toxicology, and immunotoxicology: An overview

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**ABSTRACT:** Immunology, toxicology, and immunotoxicology are three different fields of science. Immunology is the branch of science that deals with the studies of the immune system. On the other hand, toxicology is the branch of science that deals with chemicals, toxic substances, or polluted environments and their ill effect on living creatures as well as the environment. Immunotoxicology deals with both fields of immunology and toxicology. Immunotoxicology is an active area of toxicology, but this is still a relatively small area. Over the past 30 years, the main focus of immunotoxicology has been the aspects of immunotoxicity from a mechanistic or regulatory process.

***KEYWORDS:*** immune system; toxic effect; immunotoxicology; immunology; toxicology

## 1. Introduction

Immunology is the field of science that discusses the immune system. The immune system plays a very important role in our body. It protects our body from external poisonous agents, attacks of viral and bacterial cells, or any kind of defense act. The toxic agents may be chemicals, drugs, or any antigens. Therefore, studies in immunology are very important in the medical and biological fields of science. Prevention is better than cure, so for everybody we desire a healthy immune system[1,2]. A strong immune system can protect us from any kind of external toxicity, but if the immune system has not worked properly, then flu diseases and cancers can happen. If the immune system attacks your own body part instead of foreign viral or bacterial cells, then autoimmune disease occurs, which has no cure yet. So, studies of the immune system have been very important in recent decades. Immunity is of three different types (**Figure 1**).

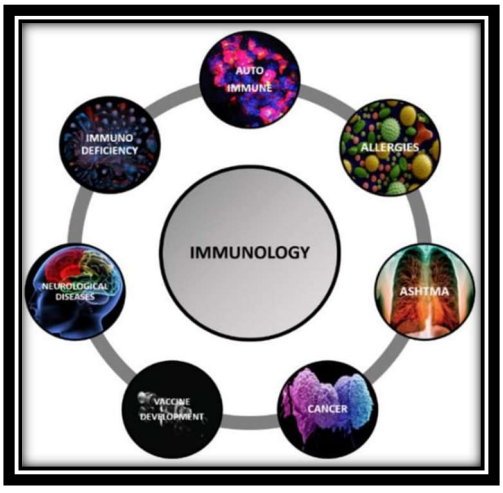


**Figure 1.** Types and domains of immunology[1].

Innate, adaptive, and passive. Innate immunity refers to inborn immunity, and adaptive immunity means the immunity that is acquired and is mainly found in vertebrates. Passive immunity forms when a person is given antibodies from the outer sources and not generated by their own body (**Figure 2**).

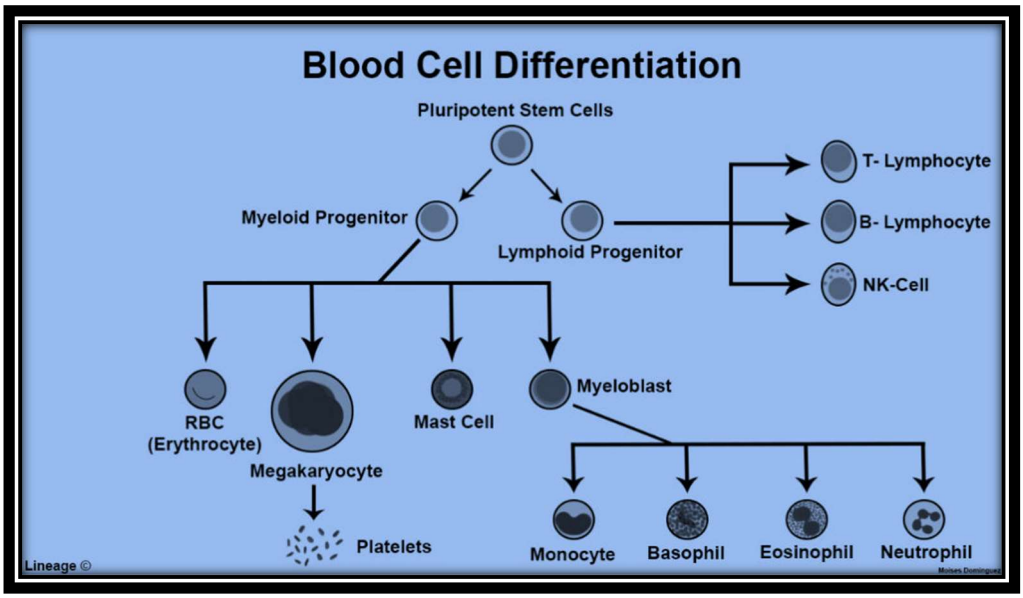


**Figure 2.** The cells which are responsible for immunity in the human body[3].

In contrast to immunology, toxicology is the knowledge of the harmful effect of man-made chemicals on natural creatures or nature. Toxicology has been divided into many segments[4–6]. These are toxicogenomics, aquatic toxicology, chemical toxicology, clinical toxicology, ecotoxicology, environmental toxicology, forensic toxicology, and medical toxicology.

Toxicogenomic[7] is the field of science that deals with the toxic effects of genes or particular cells in response to poisonous substances. Aquatic toxicology, as the name implies, is the toxic effect of the chemicals on aquatic substances. Chemical toxicology studies the effect of chemical agents on biological systems. Clinical toxicology is the field of toxicology that studies the research, precaution, and treatment of studies due to chemicals, drugs, or toxins. Ecotoxicology deals with the adverse effects of toxic chemicals or drugs on the ecosystem. Environmental toxicology is the field that studies the effect of chemical, biological, and physical substances on the environment's organisms. Forensic toxicology is the analysis of biological samples for poisonous substances or drugs. Medical toxicology is the study of different drugs for the prevention and treatment of toxic effects on human beings.

Immunotoxicology is made of two different types of poisonous effects. The first one is based on the immune system's ability to fight against the effect of a foreign chemical and minimize the toxic effect in the biological system. In contrast, the other one is the negative effects of chemicals on the immune system. The chemicals sometimes impair the immune ability of the body to resist infections. It is about the harmful effects of different drugs, such as immunosuppressives, immunostimulants, and chemicals, on the immune system, which can disturb the normal immune system. There are different types of immune toxic effects. Immunosuppression, immunostimulation, hypersensitivity, and autoimmunity.

The first one, immunosuppression, deals with the decrease of the efficacy of the immune system. In contrast, immunostimulants are substances that stimulate the immune system. Hypersensitivity deals with the abnormal response of the immune system to the guest's viral or bacterial cell. whereas autoimmunity is the effect of the immune system on the body's own healthy part (**Figure 3**).

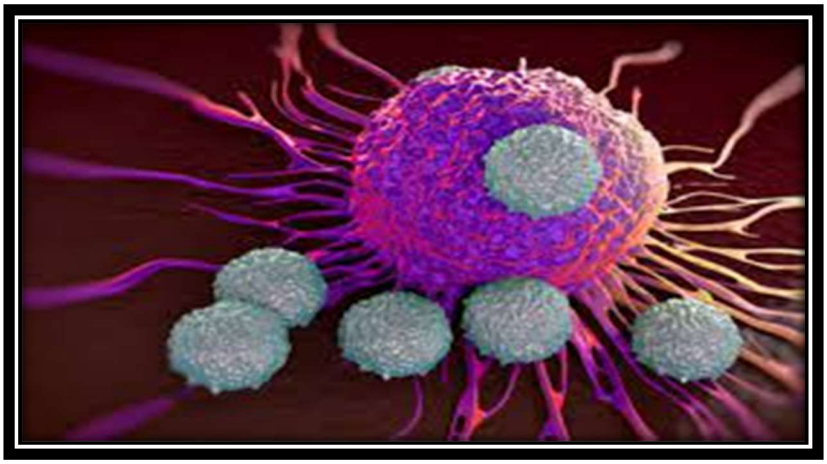

**Figure 3.** Schematic diagram for immunotoxicology[8].

## 2. Background of the fields

Immunology is a new field of science. The field was generated when Jenner invented vaccinia (1880), which protects the human body from human smallpox. On the other hand, the field of toxicology was developed by Paracelsus, who linked chemistry to medicines. The immunotoxicology field was generated in the early 1970s. The field discussed altered immune function and changes in body immunity after exposure to chemicals and drugs or any type of antigens.

## 3. Parts of the immune system

The immune system is like a defense system in the human body. The organs that take part in it are the thymus gland and spleen, and the tissues are like lymph nodes, vessels, bone marrow, and skin.

### 3.1. Bone marrow

Bone marrow generates all types of immune cells. It is generally composed of stem cells, which generally change in our body from day to day. Stem cells form lymphocytes, and two different types of cells are generated from the lymphocytes. B and T cells. B cells are mature cells, and T cells are immature and travel from bone marrow to thymus, and they get mature there.

### 3.2. Thymus gland

The thymus gland is present just above the heart and behind breastbones. It is the main organ of the body to form the immune cells.

### 3.3. Lymph nodes

Lymph nodes contain a large number of immune cells. Lymph nodes are present in the opening door of the body, like in the mouth and in the genital part. Such as tonsils, adenoids, and Peyer's patches. Throughout the body, lymph nodes are there, and after infection, they start to swell and generate immune cells.

### 3.4. Vessels

Vessels are two different types: blood vessels and lymphatic vessels. Blood vessels contain lymph, which is full of immune cells. If the body is attacked by pathogens, then the immune system is activated to fight against it. The lymphatic fluid generates lymph nodes for filtering the blood. Lymphatic vessels contain filtered blood and carry it forward to the heart via the thoracic cage.

### 3.5. Spleen

The spleen is the largest immune organ in the body. It contains immune cells, which can filter pathogens, especially bacterial cells.

### 3.6. Skin

Skin is also a protective part of our body that protects us from infections and covers the entire body part.

### 3.7. Chemical toxicity

There are some chemicals that are highly toxic in nature, such as arsenic trioxide, chlorine, hydrogen cyanide, nitrogen oxide, phosphate, potassium cyanide, sodium arsenate, and sodium cyanide. Some solvents that are also highly toxic are toluene, benzene, and all chloro solvents like chloroform, dichloromethane, methanol, etc.

### 3.8. Drug toxicity

Some drugs have maximum side effects but have to be provided in need. People should be aware of it to minimize its use. Allergic medicines like diphenhydramine have severe side effects like dry mouth, cough, and constipation. Some medicines, like warfarin, create problems in bleeding complications. Other medicines like lisinopril (Prinivil), captopril (Capoten), enalapril (Vasotec), and ramipril (Altace) have side effects like kidney failure and ultimate death.

Biguanides such as metformin are common medicines for patients with diabetes with side effects of low blood pressure and low body temperature.

Antibiotics levofloxacin and ciprofloxacin are fluoroquinolones. These medicines were to treat urinary tract infections and bronchitis. The side effects are severe, like tendon injuries, ruptures, or tears of the aorta.

Painkillers such as acetaminophen (Tylenol) and non-steroidal anti-inflammatory drugs such as ibuprofen (Motrin, Advil) and naproxen (Aleve) are used commonly. The common side effects are bleeding in the stomach, ulcers, kidney damage, high blood pressure, and chances of heart attack.

## 4. Conclusion

Immunology and toxicology relations are very significant in today's date. The autoimmune diseases[9–11] (164 diseases, to the best of our knowledge) are increasing day by day, which are caused by misleading of the immune system. The immune system is the police force of the body. If any foreign cell attacks the body immediately, the immune system starts working to protect it, and if the immune system stops reacting, then the body will soon be attacked by several diseases and cause ultimate death. So, a healthy immune system is very important to leading a healthy life. Therefore, the study of immunology, toxicology, and immunotoxicology has tremendous importance in present and future days; otherwise, the existence of human lives is in danger in the future era of modern technologies.

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## Conflict of interest

The author declares no conflict of interest.

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Perspective

# Perspective: Senselessness in ecotoxicological investigation

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**ABSTRACT:** Whether openly stated or not by the authors of nearly all ecotoxicology studies published in the peer-reviewed literature, the studies are conducted with the thinking that the furnished information is valuable for the field of ecological risk assessment. Reasonably too, those reading these published works share the same sentiment. These situations are unfortunate, for a closer inspection of the research conducted reveals that commonly, one or more study aspects render the data generated to be not utilizable for ecological risk assessment purposes. Some frequently encountered complications include using test species that are never assessed for health effects in the wild, the mode of chemical dosing deviating radically from the manner in which actual chemical exposures occur, and lacking an assessment methodology for expressing health impacts. Because ecotoxicological investigation often does not align with the applied-science needs of ecological risk assessment, this article wonders why the studies proceed. Moreover, this article recommends that authors caution their readership about the limited or lacking utility of the research they describe in the area of fostering assistance and embellishment to ecological assessment science.

***KEYWORDS:*** ecotoxicology, ecological risk assessment, research, contaminants, chemicals

## 1. Introduction

As toxicologists and toxicology enthusiasts, I trust it is our solemn dream to see our science continue to grow. While thousands of studies have already been conducted and published to date over decades, we know there are still thousands more waiting to unfold. Through amassing knowledge in our chosen field and applying it in conjunction with cues we take from the environment, we commonly find ourselves directed to formulate new questions to explore. It would seem then that I am supporting the case for endless toxicological investigation reigning supreme. I am in fact doing just that, albeit with one rather critical qualification; I see no need for ecotoxicological study to continue, and this contention is supported by reliable and readily available science.

## 2. An absent need for ecotoxicological study: Absence of impacts in the field

Although ecotoxicology, like any research discipline, has its pure and applied components, for all intents and purposes, ecotoxicology studies are of the latter genre. With only minor exceptions, the studies are used only to support the field of ecological risk assessment (ERA)—the concern that plants and animals living in contaminated terrestrial and aquatic locations will develop health effects or die. It is here, though, that the well-intended nature of the many studies we know of and continue to read about breaks down. Since ERA's inception some four decades ago, there has yet to be a singular instance

of an ecological receptor bearing signs of stress, illness, disease, or population decimation due to the chemical footprint left behind by those who accidentally or deliberately contaminated a local environment[1]. Given this reality, one that is undoubtedly difficult for would-be research ecotoxicologists to absorb, there is no purpose served in chemically dosing animals (e.g., via intubation, injection, etc.) or exposing them to contaminated environmental media (e.g., earthworms placed into jars of amended soil, fish placed in contaminated aquaria, etc.). If animals and plants in the wild bear no signs of having toxicologically succumbed to the chemically contaminated media with which they live, why perpetuate the myth that additional study is needed to afford species with health protection? To put it very simply, animals and plants at contaminated sites do not need our help; over multiple decades of chemical exposure, they have demonstrated their keen resilience[2]. They are functioning well, providing the ecosystem functions they should, and most importantly, are perpetuating their own while doing so.

## 3. Ecotoxicological studies fail to furnish useful information

At this early point, it's also worth noting that the design of classical/conventional ecotox studies leaves much to be desired. The field of ecotoxicological research has yet to recognize that there are no environmental science or ERA gains to be had when testing the standard way. Thus, testing chemicals alone for the effects they might pose, when contaminated sites are almost always present with a chemical suite, is a wasted endeavor. Injection and intubation as modes of chemical delivery are not replacements for the natural dietary inputs that animals in the wild experience. The fixed ambient temperatures and supplied artificial laboratory lighting for caged animals that have never lived in the outdoors in no way mimic the environmental settings of the animals in the wild that might concern us. And the list of disconnects between the imposed chemical exposures of laboratory and mesocosm studies and the actual chemical exposures occurring in nature goes on.

We would do well to review several recurrent ecotoxicology study types, including those pertaining to the earlier-mentioned pure science arena, for they will secure the overarching point that we are generating data for which no valid need exists. A first example is that of amending jarred soils with varying concentrations of explosives and tracking several endpoints (e.g., survival, growth, reproduction) in exposed soil invertebrates such as earthworms, collembolans (i.e., springtails), and enchytraeids (i.e., potworms). These animal groups are virtually never collected from the field at contaminated sites and are never health-assessed in ERAs. More to the point, ERA guidance does not exist for these groups, and realistically, there is no anticipation that springtail and potworm protection concerns will someday serve as triggers for hazardous waste site remedial actions. It should be noted too that fueling the drive to ascertain supposed protective soil concentrations for springtails and potworms is the argument that sizeable accumulations of explosive residues occur at military test ranges. The reality is that due to safety concerns, down-range soil sampling can't ever proceed for the most part. Thus, we don't truly know that sizeable explosive accumulations actually occur, and we can never know if soil invertebrates in down-range areas are at risk. Again, what need is there for developing toxicological benchmarks for soil and litter invertebrates[3]? For a given soil contaminant, why would anyone need to know if potworms develop toxicological endpoints sooner than do springtails, or vice versa?

Ecological risk assessors know that the contaminant inhalation pathway is never assessed for any ecological receptor group (e.g., mammals). While it is true that chronic mammal inhalation studies do not occur and no formal methods exist for the assessment of the mammal inhalation pathway, there is a much simpler explanation for ERA's non-attention to ecological receptor inhalation risk. Plainly, ERA recognizes that in terms of contribution to overall risk, inhalation plays a relatively minor role, and to the

point that ERA uncertainty sections routinely avow the same. Why, then, are there occasional research attempts to quantify the chemical inhalation exposures of fossorial mammals[4–6], even if we ignore artificially constructed tunneling made of flexible irrigation hose likely bearing little or no resemblance to the actual burrowing systems that small mammals construct?

The dermal contaminant uptake route is also never evaluated in ERAs, and legitimate defenses for the pathway's non-consideration take two forms. First, ecological receptors have relatively impervious integuments that greatly limit dermal transfer (e.g., a thick fur coat). Second, ongoing preening behavior converts would-be dermal transfer events to chemical uptake events via ingestion. Here it is fair to ask why certain focused research occasionally proceeds wherein human placental tissue is suffused with a contaminant-bearing aqueous solution, with the goal of estimating a bird's chemical uptake through its foot pads. Realistically, with the mounds of uncertainty associated with a study design like this, can such research produce helpful information to support ERAs? Does anyone honestly think that more research of this kind will lead to the dermal uptake route coming to be regularly assessed for birds in ERAs in the future?

## 4. Senselessness in ecotoxicological investigation

Fairly, senselessness in ecotoxicological investigation today might be epitomized by the research efforts that proceed for perfluoroalkyl and poly-fluoroalkyl substances (PFAS). As of this writing, distinct PFAS forms that for more than a half-century have taken up residence in matrices of every kind, number close to 1500, and there is every reason to suspect that the count of these 'forever chemicals' will continue to climb. With PFAS having been detected in the blood of 97% of Americans[7], there is no question that PFAS residues are present as well, in all animal tissues everywhere. While it is understandable that the PFAS-ecotox investigation took off once the toxicity of the family of compounds was publicly established in the late 1990s, it is fair to take the ongoing investigation to task, asking if serviceable ecotoxicological information can possibly be brought forward at this late date. Whatever the testing scheme and whatever the species studied, from where is a researcher to procure his/her experiment controls (i.e., animals that have not been exposed to one or more PFAS forms and that do not bear a PFAS body burden)? Does it make sense to dose birds with a singular PFAS form so that a toxicity reference value (TRV) can subsequently be derived for it when birds in the wild are exposed to tens of PFAS forms at a time, in addition to numerous other environmental contaminants? Does it make sense to develop PFAS form-specific TRVs while the list of PFAS forms is still growing? Why are researchers publishing books on PFAS TRVs and couching such TRV information within a risk context[8] when these hard-hitting challenges to ecotoxicological investigation abound? And one more hard-hitting question: Assuming there are workarounds for the many confounding factors at play while conducting PFAS ecotoxicological investigation, should study outcomes point to birds everywhere being health-imperiled? Have we ways to retract PFAS from environmental matrices to ameliorate the dismal projections? The answer is, of course not. What utility lies in the PFAS ecotoxicological investigation?

There is more; through the novelty of developing TRVs for PFAS, scientists and risk assessors are likely to forget that the hazard quotients (HQs) computed from TRV usage are not risk measures altogether[9,10]. This brief treatment on PFAS then puts the spotlight on an overarching problem for ERA that ecotoxicological investigation only fosters, namely giving the impression that such investigation is assisting ERA when in fact, no ERA inroads are being made at all.

## 5. Cautionary notes

Assuming that animal care and use protocols are followed in ecotoxicological investigations, surely there are no illegalities at play. So long as researchers are able to assemble the resources they need to conduct their studies of interest, they may do as they please. There are, though, other considerations that could make for cases of 'wrongdoing' in the ecotoxicology arena. The potential is high for those working in the applied field of ERA to be misled, looking at newly published ecological findings as constituting a windfall for the development of the ERA science they embrace. Researchers, though, have a responsibility to let their readership know that due to the particulars of their studies (e.g., the choice of test species, the way in which a study has been conducted), their work is not adaptable to ERA in the main. By way of example, research ecotoxicologists who unveil bioaccumulation/biouptake data for any species and for any somatic compartment need to caveat their work, reminding their audience that, still to the present day, means do not exist for equating chemical body burdens with health effects in the animals that have them. In a similar way, researchers need to openly state other critical points in their works, such as a) the species they have elected to work with being one that is never being assessed in ERAs (to include any amphibian, reptile, or soil microbe), and b) their tested species lacking ecological protection concerns (e.g., insects), unless it should be classified as being of special status. It is hoped that ecotoxicological investigation of the future will only involve species we truly aim to protect (or their reasonable surrogates), and that good sense is exercised in research protocols.

## Conflict of interest

The author declares no conflict of interest.

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