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RESEARCH ARTICLE

Qualitative and Quantitative *In Silico* Assessment of Acute Toxicity of PFOA and PFOS

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Qualitative and Quantitative In Silico Assessment of Acute Toxicity of PFOA and PFOS

SUMMARY

Perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) have been categorized as persistent organic pollutants under the Stockholm Convention. Moreover, the IARC classifies them as classified carcinogens (Group 1) and possibly carcinogenic (Group 2B) to humans, respectively. Toxicological and epidemiological evidence links PFOA and PFOS to multiple adverse health outcomes, including renal and testicular cancer. However, data on their acute toxicity remain limited. To address this gap, this study performed a comprehensive in silico assessment of PFOA and PFOS using both qualitative and quantitative computational models. Acute toxicity was evaluated for oral, dermal, inhalation routes, eye, and skin exposure. Qualitative predictions from Toxtree, STopTox, admetSAR, and ADMETlab consistently indicated toxicity, particularly via oral and inhalation exposure, as well as through eye and skin contact. The carboxylic acid moiety was highlighted as a key toxicophore in PFOA, while the whole structure was identified as a toxicophore in PFOS, possibly contributing significantly to their toxicity. Quantitative analyses, employing ProTox-3.0, Deep-PK, ToxACoL, TEST, VEGA, and QSAR Toolbox, revealed notable variation in LD50 estimates, with LC50 data being unavailable. Overall, LD50 predictions supported high acute toxicity via oral exposure. These findings underscore the acute toxicity potential of PFOA and PFOS and additionally demonstrate the value of in silico toxicology for screening data-poor chemicals and supporting regulatory decision-making. Although variability among prediction algorithms and training datasets presents limitations, in silico approaches offer a rapid and cost-effective hazard assessment. Future studies should focus on improving predictive performance through expanded datasets and integration with experimental validation.

Keywords: *PFOA, PFOS, acute toxicity, in silico toxicology.*

PFOA ve PFOS'un Akut Toksisitesinin İn Siliko Nitel ve Nicel Değerlendirilmesi

ÖZ

Perflorooktanoik asit (PFOA) ve perflorooktan sülfonik asit (PFOS), Stockholm Sözleşmesi kapsamında kalıcı organik kirleticiler (POP) olarak sınıflandırılmıştır. Ayrıca IARC, bu maddeleri sırasıyla insanlar için kesin karsinojen (Grup 1) ve muhtemelen karsinojen (Grup 2B) olarak değerlendirmektedir. Toksikolojik ve epidemiyolojik kanıtlar, PFOA ve PFOS'u böbrek ve testis kanseri dahil olmak üzere birçok olumsuz sağlık sonucuyla ilişkilendirmektedir. Ancak, bu maddelerin akut toksisitesine ilişkin veriler sınırlıdır. Bu boşluğu doldurmak amacıyla, bu çalışma PFOA ve PFOS'un nitel ve nicel hesaplamalı modeller kullanılarak kapsamlı bir in siliko değerlendirmesini gerçekleştirmiştir. Akut toksisite oral, dermal, inhalasyon yolları, göz ve cilt maruziyeti açısından değerlendirilmiştir. Toxtree, STopTox, admetSAR ve ADMETlab tarafından yapılan nitel tahminler, özellikle oral ve inhalasyon yoluyla, ayrıca göz ve deri temasında toksisiteyi tutarlı biçimde göstermiştir. PFOA için karboksilik asit grubunun temel bir toksikofor olduğu, PFOS için ise tüm yapının bir toksikofor olarak belirlendiği ve bunun toksisiteye önemli ölçüde katkı sağlayabileceği görülmüştür. ProTox-3.0, Deep-PK, ToxACoL, TEST, VEGA ve QSAR Toolbox kullanılarak yapılan nicel analizler, LD50 tahminlerinde kayda değer değişkenlik olduğunu, LC50 verisinin ise bulunmadığını göstermiştir. Genel olarak, LD50 tahminleri oral maruziyet yoluyla yüksek akut toksisiteyi desteklemiştir. Bu bulgular, PFOA ve PFOS'un akut toksisite potansiyelini vurgulamakta ve ayrıca veri açısından yetersiz kimyasalların taranması ile düzenleyici karar verme süreçlerinin desteklenmesinde in siliko toksikolojinin değerini göstermektedir. Tahmin algoritmaları ve eğitim veri setleri arasındaki farklılıklar sınırlılık oluştursa da, in siliko yaklaşımlar hızlı ve maliyet-etkin bir tehlike değerlendirmesi sağlar. Gelecekteki çalışmalar, daha geniş veri kümeleri ve deneysel doğrulamanın entegrasyonu yoluyla tahmin performansının artırılmasına odaklanmalıdır.

Anahtar Kelimeler: *PFOA, PFOS, akut toksisite, in siliko toksikoloji*

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INTRODUCTION

Perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) are synthetic per- and polyfluoroalkyl substances (PFAS) widely used globally. Their unique physico-chemical properties lead to their widespread use, along with their salts and related compounds, in numerous applications and consumer products across various sectors. The presence of a carbon–fluorine bond, one of the strongest in organic chemistry, accounts for the environmental and biological persistence, long-range environmental transport, bioaccumulation, and biomagnification characteristic of this class of chemicals (EFSA, 2008, EFSA Panel on Contaminants in the Food Chain (CONTAM) et al., 2018; UNEP/POPS/POPRC.14/6/Add.2, 2018).

Occupational exposure to PFOA and PFOS is among the highest, primarily through inhalation, with potential contributions from dermal absorption and dust ingestion. The highest occupational exposure typically occurs in fluorochemical manufacturing. For the general population, primary exposure to PFOA and PFOS occurs through diet and drinking water, with consumer products serving as an additional potential source. However, in communities situated near polluted sites, drinking water becomes the predominant exposure pathway for the general population (UNEP/POPS/POPRC.4/15/Add.6, 2008; UNEP/POPS/POPRC.14/6/Add.2, 2018).

PFOA and PFOS are detectable in blood samples of populations globally, with median levels in communities near polluted sites being up to a hundred times higher. A portion of the body burden may also originate from precursors—other PFAS that metabolize into PFOA or PFOS within the body. These compounds accumulate in various tissues, such as the blood, liver, and lung. Furthermore, they are detected in the placenta, cord blood, and embryonic tissues, and can be transferred to infants through breast milk. Both agents undergo enterohepatic recirculation and kidney reabsorption. Excretion occurs via urine and feces, with half-lives ranging from hours to months in experimental animals and on the order of several years in humans (UNEP/POPS/POPRC.4/15/Add.6, 2008; UNEP/POPS/POPRC.14/6/Add.2, 2018). PFOA and PFOS are recognized as detrimental to both human and animal health. In humans, exposure to these compounds is associated with reproductive and developmental toxicity, testicular cancer, immunological suppression, and thyroid dysfunction. Similarly, in animals, PFOA and PFOS have been linked to reduced sperm quality, immunotoxicity, and various other adverse health outcomes (Polychronidou & Nag, 2025).

Given their persistence, bioaccumulation potential, adverse effects on human health and the environment, and capacity for long-range environmental transport, PFOA, PFOS, their salts, and related compounds have been listed under Annexes A and B of the Stockholm Convention (UNEP/POPS/COP.9/SC-9/12, 2019; UNEP/POPS/COP.9/SC-9/4, 2019). Additionally, PFOA is categorized as a Group 1 carcinogen (carcinogenic to humans), and PFOS as a Group 2B carcinogen (possibly carcinogenic to humans) by the International Agency for Research on Cancer (IARC, 2025). Despite their inclusion in the Stockholm Convention and IARC classifications, acute toxicity data remain limited, partly due to analytical challenges associated with detecting and quantifying these compounds.

Considering these findings, a substantial data gap persists regarding the acute toxicity of PFOA and PFOS. Therefore, conducting *in silico* studies to assess their toxicity is essential. Such approaches align with the 3Rs principles (Replacement, Refinement, and Reduction) and offer advantages in terms of speed, safety, and alignment with innovative, non-animal testing strategies, specifically New Approach Methodologies (NAM). This study employed qualitative *in silico* methods to determine acute toxicity and identify toxicophoric groups within PFOA and PFOS struc-

tures, thereby classifying their toxicity. Following the qualitative analysis, the acute toxicity of PFOA and PFOS was quantified by estimating the theoretical median lethal dose (LD_{50}) and median lethal concentration (LC_{50}) across various exposure routes using a suite of quantitative *in silico* methods. The final stage of this research involved quantitative extrapolation of LD_{50} values from animal models to humans. The conceptual framework for these toxicological assessments is schematically illustrated in Figure 1.

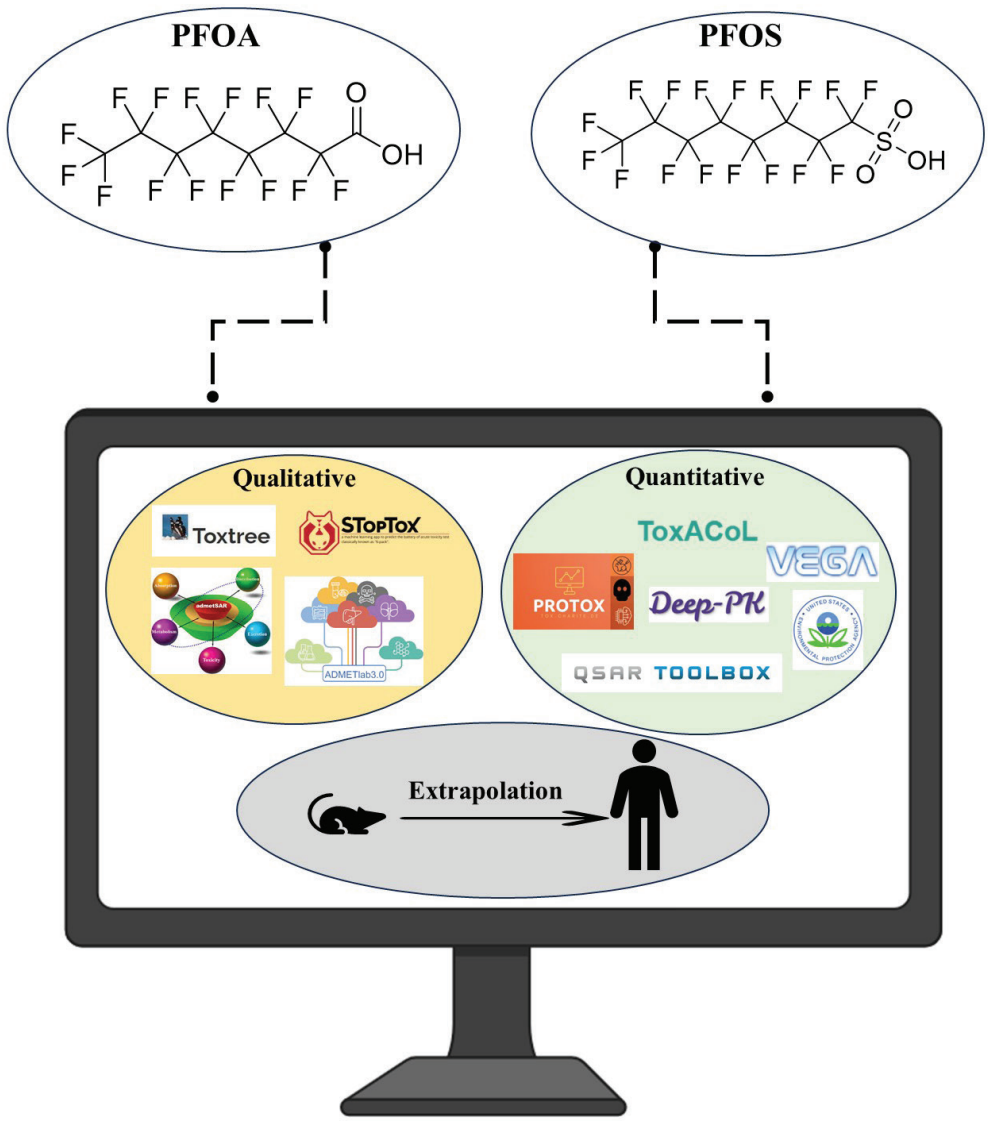


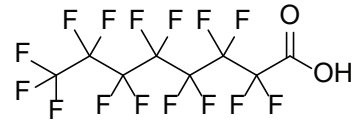
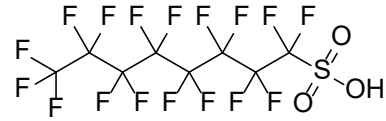
Figure 1. Overview of the *in silico* workflow used for acute toxicity prediction of PFOA and PFOS

MATERIALS AND METHODS

Investigated compounds

This study investigated PFOA and PFOS. Their Chemical Abstracts Service (CAS) numbers, Simplified Molecular Input Line Entry System (SMILES) strings, and molecular weights (MW) were obtained from the PubChem database (Kim et al., 2025). The chemical structures of the compounds were prepared using ChemDraw Professional software. All details are presented in Table 1.

Table 1. Chemical identity of PFOA and PFOS

CAS Number	Acronym	MW (g/mol)	SMILES string	Chemical structure
335-67-1	PFOA	414.07	C(=O)(C(C(C(C(C(C(C(F)(F)F)(F)F)(F)F)(F)F)(F)F)(F)F)(F)F)O	
1763-23-1	PFOS	500.13	C(C(C(C(C(F)(F)S(=O)(=O)O)(F)F)(F)F)(F)F)(C(C(C(F)(F)F)(F)F)(F)F)(F)F	

***In silico* tools for qualitative predictions**

Toxtree 3.1.0

Toxtree (https://toxtree.sourceforge.net) is a comprehensive, flexible, and user-friendly open-source software application designed to estimate toxic hazards through a decision tree approach (Patlewicz et al., 2008). It accommodates molecular structures provided in SMILES notation or as 2D chemical structures. In this study, the Revised Cramer Decision Tree method was employed, which classifies substances of known structure into Class I, II, or III based on their potential oral toxicity. Low (Class I): Substances characterized by simple chemical structures and efficient metabolic pathways, suggesting a low order of oral toxicity. Intermediate (Class II): Substances possessing structures that are less innocuous than Class I but lack the clear structural indicators of toxicity found in Class III. High (Class III): Substances with chemical structures that preclude a strong initial presumption of safety, may suggest significant toxicity, or contain reactive functional groups. Each class is associated with a specific threshold of toxicological concern, a concept defining human exposure thresholds below which negligible health risk is anticipated. The derived human exposure thresholds were 1.8 mg/day for Class I, 0.54 mg/day for Class II, and 0.09 mg/day for Class III substances (Patlewicz et al., 2008).

STopTox

Systemic and Topical Chemical Toxicity (STopTox) (available online: https://stoptox.mml.unc.edu) is a comprehensive *in silico* platform designed for the accurate prediction of six key human acute toxicity endpoints, commonly referred to as the "6-pack". These evaluations encompass acute oral, dermal, and inhalation toxicity, alongside skin and eye irritation/corrosion, and skin sensitization. The primary methodology relies on Random Forest algorithms, chosen for their demonstrated robustness in handling intricate datasets. Each model within STopTox is dedicated to specific endpoints; for instance, the acute oral toxicity model adheres to OECD guidelines and utilizes data derived from rat studies (TG 401, 420, 423, and 425). Predictions are generated using molecular descriptors, such as Molecular ACCess System keys, which encode specific molecular features to identify toxicophores-structural elements associated with toxicity (Borba et al. 2022). For the present study, SMILES strings of PFOA and PFOS were input into STopTox to obtain predictions, confidence levels, applicability domains (AD), and associated toxicophores.

admetSAR 3.0

AdmetSAR 3.0 (available online: https://lmmd.ecust.edu.cn/admetsar3/index.php) is an advanced and scientifically rigorous software tool designed to predict the ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties of chemical compounds. AdmetSAR 3.0 is reinforced by a robust foundation, comprising over 370,000 data records from more than 100,000 compounds. admetSAR3.0 is equipped with 119 ADMET property endpoints, spanning five major categories, including 18 basic

properties, 39 ADME properties, 43 human health toxicity endpoints, 16 environmental risk assessment endpoints, and 9 cosmetic risk assessment endpoints. It employs an advanced machine learning model, the Contrastive Learning-based Multi-Task Graph Neural Network, for its predictions. In admetSAR3.0, the AD is defined by ranges of six physicochemical properties, which guide its model usage. These properties are molecular weight, logP, number of atoms, number of rings, hydrogen bond acceptors, and hydrogen bond donors. AD assessment classifies results into three categories: "In Domain", "Out Domain", and "Warning". Predictions for compounds "In Domain" are deemed reliable and valid. For those marked as "Warning", caution is recommended when using the predictions. Predictions for compounds "Out Domain" are not advisable for reference (Gu et al., 2024). In the present study, the SMILES strings of PFOA and PFOS were input into the ADMET evaluation module of admetSAR 3.0 to obtain the predicted probabilities (%) of acute oral, inhalation, and dermal toxicity, as well as eye and skin irritation, for each compound.

ADMETlab 3.0

ADMETlab 3.0 (available online: https://admetlab3.scbdd.com) evaluates a wide array of endpoints, including physicochemical properties, ADME characteristics, toxicity predictions, and specific structural alerts such as toxicophores. This platform utilizes a deep learning approach, specifically a graph neural network integrated with a Directed Message Passing Neural Network framework. This methodology enables the simultaneous prediction of multiple endpoints and concurrently highlights critical structural features relevant to each prediction. In this study, SMILES strings were submitted to the ADMET evaluation section of ADMETlab 3.0 to determine the rat oral and inhalation acute toxicity, as well as eye irritation and skin sensitization potential of PFOA and PFOS. The output provides a toxicity probability score ranging from 0 to 1. Toxicity classifications were empirically defined as follows: 0-0.3 indicating low toxicity, 0.3-0.7 indicating moderate toxicity, and 0.7-1.0 indicating high toxicity (Fu et al., 2024). In this study, the results are presented as the percentage equivalents of these probability values (e.g., 0.3 = 30%, 0.7 = 70%).

***In silico* tools for quantitative predictions**

ProTox 3.0

ProTox-3.0 operates as a web-based platform (available online https://tox.charite.de/protox3/index.php?site=home), leveraging machine learning algorithms, chemical similarity analyses, and molecular fragment analysis to predict the toxicity of small molecules (Banerjee et al., 2024). It offers a comprehensive array of functionalities for assessing target organ toxicity, forecasting toxicological endpoints, and estimating LD_{50} values. The tool has undergone extensive validation, with its predictive efficacy supported by studies demonstrating high sensitivity (73.08%) and specificity (94.62%). ProTox-3.0 employs a hybrid methodology that integrates similarity-based models with fragment-based toxicity rules, thereby identifying toxicophores and structural alerts. These components contribute to overall risk evaluation by providing mechanistic insights. The acute toxicity model within ProTox-3.0 utilizes molecular fingerprints and established toxic fragments to categorize chemicals into six toxicity classes, aligning with the Globally Harmonized System (GHS) (UNECE, 2025). In this study, the SMILES strings of PFOA and PFOS were input into the "Tox-Prediction" module of ProTox-3.0 to obtain the predicted acute oral LD_{50} values (mg/kg bw) and the corresponding GHS toxicity classes.

Deep-PK

Deep-PK (accessible online: https://biosig.lab.uq.edu.au/deeppk/) represents an advanced deep learning-based platform engineered to forecast the pharmacokinetic and toxicological attributes of chemical compounds, including acute toxicity endpoints (Myung et al., 2024). The system effectively employs graph neural networks and molecular graph fingerprinting to meticulously model the intricate relationships between chemical structure and biological

activity. The architectural design of Deep-PK facilitates the prediction of 73 distinct endpoints, encompassing 64 ADMET-related properties and 9 physicochemical characteristics. Input typically involves SMILES strings or structure data files, which are subsequently converted into molecular graph representations. Through sophisticated message-passing neural networks, the system extracts spatial and topological features critical for toxicity prediction.

The model's training and validation were conducted using extensive datasets that incorporate experimentally derived toxicological data. Cross-validation studies have confirmed Deep-PK's robust predictive performance across various endpoints, achieving an average Matthews correlation coefficient of 0.71. For acute oral toxicity, the platform reports R^2 values exceeding 0.60 and root mean square errors (RMSE) ranging from 0.5 to 0.6 log units, contingent on the chemical class and specific endpoint. The platform supports batch predictions and includes model uncertainty estimates, making it a robust tool for preliminary hazard identification (Myung et al., 2024). In this study, the SMILES strings of PFOA and PFOS were input into the "Prediction" module of Deep-PK to predict rat acute oral LD_{50} values. The results were expressed as log[1/(mol/kg)]. Subsequently, the molecular weights (g/mol) of PFOA and PFOS were used to convert the values from log[1/(mol/kg)] to mg/kg bw.

ToxACoL

ToxACoL (Adjoint Correlation Learning for Multi-Condition Acute Toxicity Assessment; available online at https://toxacol.bioinforai.tech) is an advanced deep learning-based platform designed for multi-endpoint acute toxicity prediction. It simultaneously models inter-relationships among toxicity endpoints by jointly learning representations of both compounds and endpoints through an adjoint correlation learning strategy. Chemical structures, provided as SMILES strings, are used to predict acute toxicity outcomes across 13 species (e.g., mouse, rat, rabbit, and human), 8 administration routes (e.g., oral, intravenous, dermal), and 3 measurement indicators (e.g., LD_{50}). This integrative approach enables reliable cross-species and cross-route extrapolation even under data-sparse conditions. Benchmark evaluations have demonstrated that ToxACoL achieves approximately 43–87% performance improvements on human endpoints compared with conventional models, while reducing required training data by 70–80% (Lu et al., 2025). In this study, SMILES strings of PFOA and PFOS were submitted to ToxACoL to obtain predicted LD_{50} values in mg/kg.

Toxicity estimation software tool

The Toxicity Estimation Software Tool (TEST) is an open-source application developed by the US Environmental Protection Agency (EPA) to streamline and enhance the efficiency of chemical toxicity prediction. The software enables users to input molecular structures using either SMILES notation or CAS numbers, thereby simplifying the toxicity assessment process. Its most recent iteration, version 5.1.2, incorporates multiple predictive models that estimate acute toxicity through the application of structural analogues and multivariate regression techniques. This methodology improves both the efficacy and accessibility of chemical toxicity evaluations across a range of applications. TEST utilizes an extensive collection of molecular descriptors derived from the EPA ECOTOX databases, which include structural, constitutional, connectivity-based, shape-related, topological, molecular distance, fragment-based, and electrotopological features. The software's Consensus method combines predictions from various QSAR models, averaging their outputs to generate a comprehensive toxicity estimate. By integrating multiple predictive methodologies, this approach ensures that the final toxicity estimation is both balanced and dependable, taking into account the AD of each model. Among the predictive strategies implemented in TEST, the consensus method is recognized as the most accurate, as it synthesizes results from hierarchical clustering and the nearest neighbor method, thereby yielding a

well-rounded toxicity evaluation. The predictive efficacy of TEST varies depending on the chosen model. For rat oral LD_{50} values, the hierarchical clustering model achieves an R^2 of 0.578 with an RMSE of 0.650, while the nearest neighbour approach yields an R^2 of 0.557 with an RMSE of 0.656. The Consensus approach provides the most refined predictions, achieving an R^2 of 0.633 and an RMSE of 0.595, underscoring its superior predictive accuracy (US EPA, 2020). In this study, the SMILES strings or CAS numbers of PFOA and PFOS were input into the TEST software, selecting "Oral rat LD_{50}" as the endpoint. All three methods (Consensus, Hierarchical Clustering, and Nearest Neighbor) were applied to PFOA and PFOS, and the predicted acute oral rat LD_{50} values were expressed in mg/kg.

VEGA QSAR

VEGA 1.2.4 (Virtual models for property evaluation within a global architecture) is a modular and freely accessible platform developed under several European Union research initiatives, such as CAESAR and ANTARES. This system integrates multiple QSAR models and prediction engines for toxicity, ecotoxicity, and physicochemical properties. The acute oral toxicity model within VEGA is predicated on a k-nearest neighbors (KNN) algorithm, trained on meticulously curated datasets of rodent oral LD_{50} values. The platform utilizes a comprehensive AD index to evaluate the dependability of its predictions. This index quantifies the similarity between the query compound and the training set, considering parameters such as rare fragments, model coverage, and descriptor range compliance. The predictive performance of VEGA's acute toxicity model (KNN) v.1.0.0 demonstrates substantial predictive power, indicated by an R^2 value of approximately 0.58 and an RMSE of 0.57 log units. VEGA incorporates automated quality checks and flags for potential issues, including low similarity to training data, atypical fragments, and sensitivity to descriptors. All models within VEGA adhere to OECD QSAR validation principles (Roncaglioni et al., 2022). In this study, the SMILES strings of PFOA and PFOS were entered into the "Insert SMILES" field, followed by selecting the endpoint "Acute Toxicity (LD_{50}) Model (KNN)-v.1.0.0," and finally clicking "Predict" in the VEGA software. The software provides the predicted acute oral rat LD_{50} values (mg/kg) along with the AD. When a compound falls within the AD of the model, the result is considered to have good reliability; when it falls outside the AD, the result is considered to have low reliability.

QSAR toolbox

The QSAR Toolbox (version 4.7.1) is a standalone computational platform jointly developed by the OECD and the European Chemicals Agency, designed to support chemical hazard assessment through diverse (Q)SAR methodologies. The Toolbox incorporates a variety of predictive strategies, including read-across, trend analysis, and local QSAR modeling. It employs curated experimental databases to generate LD_{50} and LC_{50} estimates across oral, dermal, and inhalation exposure routes. Predictions are formulated based on structural analogues grouped into chemical categories via similarity filters and clustering algorithms. The system accepts SMILES input and facilitates categorization and subcategorization workflows. Performance metrics for local QSAR models within the Toolbox vary by endpoint but typically exhibit RMSE values ranging from 0.55 to 0.65 log units and training set coverage exceeding 80%. The Toolbox includes transparent documentation of workflows, AD checks, and flagging mechanisms for unreliable predictions (Dimitrov et al., 2016). In this study, the QSAR Toolbox was used for manual categorization and data gap filling, following the approach reported by Noga and Jurowski (2025) to estimate acute toxicity. Target endpoints are defined through the following hierarchy: human health hazards, acute toxicity, LD_{50} and LC_{50} values, oral/dermal/inhalation, and rats. The categorization approach relies on "structural similarity," ensuring that only pertinent toxicity data are considered for the compound under investiga-

tion. For qualitative endpoints, the software employs a read-across method to fill missing data, while LD_{50} (mg/kg body weight) and LC_{50} (mg/L) values serve as standard measures of toxicity.

Validation

To ensure the reliability of the applied *in silico* methods, a rigorous validation strategy was implemented. This involved comparing the predicted results with available experimental data for structural analogs of PFOA and PFOS. The validation criteria for selecting structural analogs involved the use of the Generalised Read-Across (Gen-RA) tool (available online at: https://comptox.epa.gov/genra/) to identify analogs based on chemical descriptors, bioactivity descriptors, or a combination of both (hybrid approach). For this purpose, the CAS numbers of PFOA and PFOS were entered into Gen-RA, which filtered the analogs according to acute toxicity data, Morgan fingerprints, PFAS classification, and ToxCast data (Patlewicz & Shah, 2023). The structurally most similar analogs selected were perfluorodecanoic acid, ammonium perfluorooctanoate, perfluorobutane sulfonic acid, and lithium perfluorooctanesulfonate. Additionally, their predicted LD_{50} or LC_{50} values were assessed using in silico tools, and a prediction similarity index was calculated to quantify the degree of agreement between the *in silico* estimated LD_{50} or LC_{50} values and their experimentally determined reference counterparts (Patlewicz et al., 2014).

Quantitative extrapolation of LD_{50} from animals to humans

An allometric scaling approach was used to extrapolate rat LD_{50} values to human equivalent doses (HEDs). Interspecies dose conversion was performed using body surface area (BSA)-based scaling principles. Human equivalent doses were calculated according to the following equation:

HED (mg/kg)= Anmial dose (mg/kg) x Km (animal)/Km (human)

where Km represents the species-specific correction factor derived from body surface area normalization. Based on the guidance of the U.S. Food and Drug Administration (2005), the Km value is 6 for rats and 37 for adult humans. Accordingly, rat LD_{50} values were converted to HEDs by dividing the rat dose by 6.2 ($37/6 \approx 6.2$).

This BSA-based interspecies scaling method is widely applied for translating animal toxicity data to human dose equivalents (Nair & Jacob, 2016; Noga & Jurowski, 2025).

RESULTS AND DISCUSSION

PFOA and PFOS, members of the PFAS class commonly referred to as "forever chemicals," have been listed under Annexes A and B of the Stockholm Convention due to their environmental persistence, bioaccumulation, and toxicity (UNEP/POPS/COP.9/SC-9/12, 2019; UNEP/POPS/COP.9/SC-9/4, 2019). Additionally, PFOA is categorized as a Group 1 carcinogen and PFOS as a Group 2B carcinogen for humans (IARC, 2025). However, acute toxicity data remain scarce, with only one reported LD_{50} value available for PFOS (EFSA, 2008). In alignment with the 3Rs and NAM, this study utilized *in silico* toxicology tools to address these data gaps. In the present study, the acute toxicity of PFOA and PFOS was assessed using both qualitative and quantitative *in silico* methods (Figure 1.).

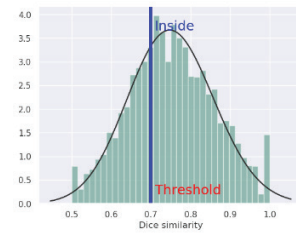
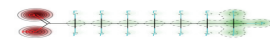
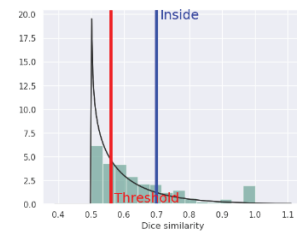
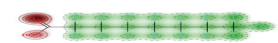
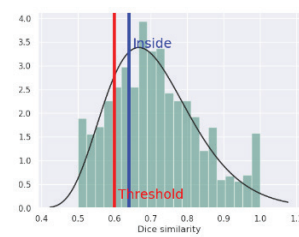
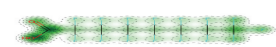
Qualitative predictions of acute toxicity for PFOA and PFOS

Table 2 summarizes the qualitative *in silico* predictions of acute toxicity for PFOA and PFOS, including toxicity classification by Toxtree and predictions for oral, inhalation, and dermal exposure routes using STopTox. The Toxtree classification of PFOA and PFOS was performed using the Revised Cramer Decision Tree system. Both compounds were categorized as Cramer Class III (high toxicity), indicating a high toxicity potential based on their structural features or the presence of reactive functional groups. STopTox predicted oral and inhalation toxicity for PFOA with high confidence values (oral: 76%, inhalation: 67%),

and oral toxicity for PFOS with a high confidence value (oral: 98%); all predictions were within the applicability domain (AD). The carboxylic acid group (–COOH) was consistently identified as the primary toxicophore for PFOA, associated with the formation of reactive acyl glucuronides, known mediators of idiosyncratic toxicity (Sawamura et al., 2010). The presence of such functional groups may enhance interactions with biological macromolecules and influence biotransformation processes.

For PFOS, the toxicophore region encompassed the entire molecular structure, including the sulfonic acid group and the perfluorinated carbon chain, reflecting the strong structural contribution of the fluorinated backbone to biological activity. The high degree of fluorination contributes to increased chemical stability, persistence, and bioaccumulation potential, which are characteristic toxicological features of PFAS (Cousins et al., 2020). These findings are consistent with reported PFAS toxicity mechanisms, which include PPARα activation as well as PPARα-independent mechanisms, such as increased oxidative stress, dysregulation of mitochondrial function, and inhibition of gap junction intercellular communication (ATSDR, 2021; DeWitt, 2015).

Table 2. Qualitative *in silico* prediction of acute toxicity of PFOA and PFOS using Toxtree and STopTox

Toxtree		STopTox				
Compounds		**Type of acute toxicity**	**Prediction**	**Confidence (%)**	**Applicability domain**	**Predicted toxicophore(s)**
PFOA	High (Class III)	oral	**Toxic** (+)	76		
		inhalation	**Toxic** (+)	67		
		dermal	**Non-Toxic** (-)	91		

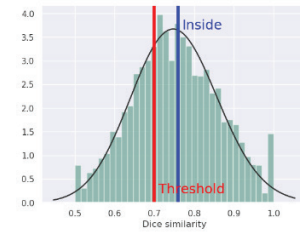
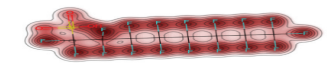
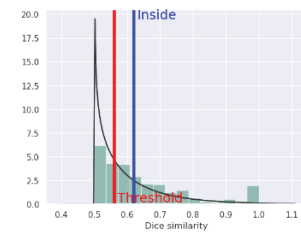
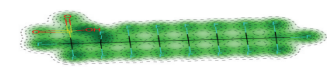
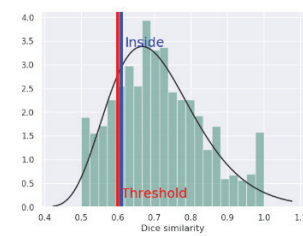
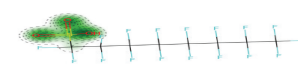
Compounds	Toxtree	STopTox				
		Type of acute toxicity	Prediction	Confidence (%)	Applicability domain	Predicted toxicophore(s)
PFOS	High (Class III)	oral	**Toxic (+)**	98		
		inhalation	**Non-Toxic (-)**	67		
		dermal	**Non-Toxic (-)**	77		

Table 3 presents the qualitative *in silico* predictions of acute toxicity for PFOA and PFOS obtained using admetSAR 3.0 and ADMETlab 3.0. admetSAR predicted high toxicity via dermal exposure (PFOA: 81.6% and PFOS: 72.4%), eye exposure (eye irritation for PFOA: 98% and PFOS: 95.8%; eye corrosion for PFOA: 92.7% and PFOS: 90.2%), and skin irritation for PFOA (72%). PFOS showed a moderate potential for skin irritation (59.6%), and both compounds exhibited a moderate potential for skin sensitization. Predictions for acute oral toxicity indicated moderate toxicity for PFOA (42.1%) and a lower likelihood of toxicity for PFOS (17.8%), although all predictions carried an "AD warning" flag, suggesting that these results should be interpreted with caution.

ADMETlab 3.0 predicted that PFOA and PFOS are highly toxic via eye exposure (eye irritation for PFOA: 98.8% and PFOS: 97.9%; eye corrosion for PFOA: 95.5% and PFOS: 93.3%) and toxic via skin exposure (skin sensitization for PFOA: 74.8% and PFOS: 68.8%). Predictions for acute oral toxicity were moderate (PFOA: 41.6% and PFOS: 38.2%). Additionally, AD results were not reported in ADMETlab 3.0.

When comparing AD results, STopTox provided the most reliable qualitative toxicity assessment, consistently indicating oral toxicity for both compounds and inhalation toxicity for PFOA, whereas admetSAR and ADMETlab additionally highlighted eye and skin exposure risks (Tables 2 and 3.).

Table 3. Predicted acute toxicity potential of PFOA and PFOS using admetSAR and ADMETlab

	admetSAR 3.0 -- Probability (%)		**ADMETlab 3.0 -- Probability (%)**	
Type of toxicity	**PFOA**	**PFOS**	**PFOA**	**PFOS**
acute oral toxicity	42.1	17.8	41.6	38.2
acute inhalation toxicity	N/A	N/A	N/A	N/A
acute dermal toxicity	81.6	72.4	N/A	N/A
eye irritation	98.0	95.8	98.8	97.9
eye corrosion	92.7	90.2	95.5	93.3
skin irritation	72.0	59.6	N/A	N/A
skin sensitization	44.1	53.7	74.8	68.8

PFOA: Perfluorooctanoic acid, *PFOS:* Perfluorooctane sulfonic acid, *N/A*: Not Available

Qualitative tools identified oral and inhalation exposures as primary toxicity routes, with additional potential for eye and skin exposures. Moreover, structural toxicophore assessment consistently implicated the carboxylate moiety as the principal reactive feature in PFOA, and the entire molecular structure in PFOS (Tables 2 and 3.). The carboxylate moiety is known to undergo metabolic activation to form acyl-glucuronides, which are metabolites associated with idiosyncratic toxicity in pharmaceuticals, highlighting a mechanistically plausible pathway for PFOA-mediated adverse effects (Sawamura et al., 2010).

Although AD parameters were reported separately for each *in silico* tool, an overall evaluation of model reliability is particularly important for highly fluorinated compounds such as PFOA and PFOS. These perfluorinated substances exhibit distinctive physicochemical characteristics, including extensive fluorination, exceptional chemical stability, and atypical partitioning behavior, which may limit their representation in conventional QSAR training datasets (ATSDR, 2021). In the present study, reliability was supported by an acceptable AD status and consistency of toxicity classification across multiple platforms. Nevertheless, due to the structural uniqueness of PFAS, the predictions should be interpreted with appropriate scientific caution.

Quantitative predictions of acute toxicity for PFOA and PFOS

Quantitative acute toxicity assessments were performed using ProTox-3.0, Deep-PK, ToxACoL, TEST,

VEGA, and QSAR Toolbox, which predicted acute LD_{50} (oral and dermal) and LC_{50} (inhalation) values in rats.

Table 4 presents the theoretical LD_{50} values for rats following oral exposure to PFOA and PFOS, along with the corresponding prediction probability indices. Dermal LD_{50} and inhalation LC_{50} values were not available from any of the tools. The absence of inhalation toxicity predictions is primarily attributed to limitations in database coverage and training datasets for inhalation toxicity endpoints, particularly for highly fluorinated compounds. Therefore, the lack of inhalation toxicity predictions should be interpreted as a methodological limitation rather than an indication of safety.

Table 4. Quantitative *in silico* predictions of acute oral toxicity in rats for PFOA and PFOS, calculated using ProTox-3.0, Deep-PK, ToxACoL, TEST, VEGA, and QSAR Toolbox

	Acute oral toxicity as LD_{50} (mg/kg)								
Compounds	ProTox-3.0	SC (%)	Deep-PK	ToxACoL	TEST	SC	VEGA	RI	QSAR Toolbox
PFOA	518	100	521.5	337.6	176.4	0.6<SC	414.1	Good	N/A
PFOS	154	100	244.8	433.7	N/A	N/A	151.1	Good	561

PFOA: Perfluorooctanoic acid, *PFOS:* Perfluorooctane sulfonic acid, *N/A:* Not Available, *SC:* Similarity Coefficient, *RI:* Reliability Index.

ProTox-3.0 predicted LD_{50} values of 518 mg/kg bw for PFOA and 154 mg/kg bw for PFOS, with a high similarity coefficient (SC) of 100%, indicating high confidence in their classification as "Class IV: Harmful if swallowed ($300 < LD_{50} \leq 2000$)" for PFOA and "Class III: Toxic if swallowed ($50 < LD_{50} \leq 300$)" according to the GHS.

Deep-PK produced acute oral LD_{50} values of 521.5 mg/kg bw for PFOA and 244.8 mg/kg bw for PFOS. Both the predictions and the molecular weights of PFOA and PFOS were within the training range, indicating that the results were reliable. Both ProTox-3.0 and Deep-PK consistently indicated that PFOS is more acutely toxic than PFOA via the oral route. ToxACoL predicted that both compounds belong to GHS Class IV.

TEST provided an LD_{50} value only for PFOA (176.4 mg/kg bw) with a low SC, corresponding to GHS Class III. QSAR Toolbox provided an LD_{50} value only for PFOS (561 mg/kg bw) and classified it as GHS Class IV. VEGA predicted LD_{50} values of 414.1 mg/kg bw for PFOA (GHS Class IV) and 151.1 mg/kg bw for PFOS (GHS Class III), with a good reliability index (RI). VEGA also indicated that PFOS was more acutely toxic than PFOA.

Overall, the predicted LD_{50} values for acute oral toxicity in rats varied across the different *in silico* models, likely reflecting differences in computational methodologies and training datasets. An experimental mean acute oral LD_{50} value of 251 (199–318) mg/kg bw for PFOS was previously reported based on a single gavage administration to CD rats in a technical report by Dean et al. (1978), as reviewed in the EFSA report (EFSA, 2008). When the predicted acute oral LD_{50} values for PFOS from the *in silico* tools were compared with this experimental value, the prediction similarity percentages, from lowest to highest, were as follows: QSAR Toolbox (44.7%), ToxACoL (57.8%), VEGA (60.2%), ProTox (61.3%), and Deep-PK (97.5%). TEST did not provide an LD_{50} estimate for PFOS.

The predicted LD_{50} values were subsequently extrapolated to human toxicity estimates via allometric scaling, a widely accepted approach that accounts for interspecies differences based on body surface area (BSA). Rat-to-human extrapolation followed established guidelines for dose conversion between animals and humans (Nair & Jacob, 2016). Specifically, rat LD_{50} values were converted to human equivalent

doses (HEDs) by applying the standard interspecies conversion factor of 6.2, derived from species-specific Km values (rat Km = 6; human Km = 37), as described in the guidance of the U.S. Food and Drug Administration (2005).

Although this approach was originally developed for extrapolating NOAEL-based doses in repeated-dose toxicity studies, it has been used in the literature for approximate interspecies scaling of acute toxicity parameters (Noga et al., 2023; Noga et al., 2024; Noga & Jurowski, 2025). However, LD_{50} represents a complex endpoint influenced by toxicokinetic and toxicodynamic factors, and BSA-based scaling does not fully account for interspecies differences in metabolism, absorption, distribution, or target-organ sensitivity. Therefore, the calculated HED values should be interpreted as theoretical approximations for comparative toxicological assessment rather than definitive predictions of human acute lethality. The extrapolated human toxicity estimates derived from these computational predictions are presented in Table 5.

The extrapolation analysis of quantitative *in silico* results highlights variability in the predicted toxicity of PFOA and PFOS across computational models. Despite differences in absolute LD_{50} values, all tools consistently indicated oral toxicity. Considering predictions with higher reliability indices (SC, RI, and prediction similarity), results from VEGA, ProTox-3.0, and Deep-PK suggest that PFOA is classified as toxic if swallowed ($50 < LD_{50} \leq 300$ mg/kg), whereas PFOS is classified as fatal if swallowed ($5 < LD_{50} \leq 50$ mg/kg) (Table 5.).

Table 5. Quantitative extrapolation of acute toxicity from rats to humans based on estimated toxicity values

Compounds	Extrapolated Acute Oral Toxicity (LD_{50}, mg/kg bw)					
	ProTox-3.0	Deep-PK	ToxACoL	TEST	VEGA	QSAR Toolbox
PFOA	83.5	84.1	54.5	28.5	66.8	N/A
PFOS	24.8	39.4	69.9	N/A	24.4	90.5

PFOA: Perfluorooctanoic acid, *PFOS:* Perfluorooctane sulfonic acid, *N/A:* Not Available.

Quantitative predictions revealed model-dependent variability in LD_{50} values, yet an overall convergence toward toxic–fatal oral dose ranges. Protox-3.0, Deep-PK, and VEGA produced the most credible toxicity estimates, which aligned with limited experimental evidence. For instance, a report by Food Standards Australia New Zealand (FSANZ) indicated that PFOS and PFOA exhibited moderate acute toxicity following oral ingestion. Clinical signs in this report included hypoactivity, decreased limb tone, and ataxia, while necropsy findings comprised stomach distension, lung congestion, and irritation of the glandular mucosa (FSANZ, 2017).

Despite these findings, inter-model discrepancies underscore the need for refined algorithms trained specifically on the chemical space of PFOA and PFOS, alongside targeted experimental validation. A multi-tool integration strategy remains essential for enhancing predictive reliability and chemical risk prioritization. Therefore, the combined application of complementary computational approaches, supported by targeted experimental validation, is critical to strengthening acute toxicity assessment for PFOA and PFOS.

To validate the results, structural analogs of PFOA and PFOS were selected using Gen-RA and subjected to LD_{50} prediction through *in silico* tools. ToxACol, TEST, and QSAR Toolbox did not generate any results. Therefore, the LD_{50} predictions obtained from ProTox-3.0, Deep-PK, and VEGA, along with the prediction similarity between the experimentally determined LD_{50} values of the analogs and the predicted LD_{50} values, are presented in Table 6.

ProTox-3.0 generated results for all four analogs of PFOA and PFOS. It showed 100% prediction similarity for two analogs compared with the experimental

LD_{50} values, 83% for ammonium perfluorooctanoate, and 11% for perfluorodecanoic acid. VEGA predicted LD_{50} values for perfluorodecanoic acid and perfluorobutanesulfonic acid, demonstrating 99% prediction similarity compared with the experimental results. In contrast, Deep-PK predicted LD_{50} values for the same analogs with 17% and 77% prediction similarity, respectively.

Overall, the validation results indicate that ProTox-3.0 and VEGA produced more reliable predictions than Deep-PK for PFOA and PFOS and their analogs.

Table 6. Validation results and calculation of prediction similarity for the LD_{50} values of structural analogs

Compounds	Methods	Predicted (oral, rat) LD_{50} (mg/kg)	Confidence	Experimental (oral, rat) LD_{50} (mg/kg) from Gen-RA	Prediction similarity (%)
Perfluorodecanoic acid (CAS: 335-76-2)	ProTox-3.0	518.0	Good	57	11%
	Deep-PK	331.9	ND		17%
	VEGA	56.4	Good		99%
Ammonium perfluorooctanoate (CAS: 3825-26-1)	ProTox-3.0	518.0	Good (100%)	430	83%
	Deep-PK	NA	NA		
	VEGA	NA	NA		
Perfluorobutanesulfonic acid (CAS: 375-73-5)	ProTox-3.0	430	Good	430	100%
	Deep-PK	558.8	ND		77%
	VEGA	433.8	Good		99%
Lithium (perfluorooctane) sulfonate (CAS: 29457-72-5)	ProTox-3.0	154	Good	154	100%
	Deep-PK	NA	NA		
	VEGA	NA	NA		

ND: not determined. *N/A:* Not Available.

CONCLUSION

This study presents a systematic *in silico* assessment of the acute toxicity profiles of PFOA and PFOS. Qualitative models consistently indicated toxicity through oral and inhalation routes, alongside an additional risk from ocular and skin exposure. Quantitative estimates varied across platforms, but Protox-3.0 and VEGA produced the most reliable LD_{50} predictions, as evidenced by their RI, SC, and experimental comparisons. Allometric scaling further confirmed a GHS Class II–III acute toxicity potential in humans. Notably, LC_{50} values could not be reliably assessed due to current model limitations.

These findings highlight both the value and limitations of *in silico* methods for characterizing PFOA and PFOS hazards. Future efforts should prioritize expanding model training datasets, improving AD coverage, and integrating *in vivo-in vitro* validation to enhance predictive reliability. Overall, computational toxicology offers a powerful first-tier strategy for prioritizing toxicological testing and guiding regulatory risk assessment for PFOA and PFOS.

AUTHOR CONTRIBUTION STATEMENT

F.K: Developing hypothesis, *in silico* experimental analysis and interpretation of the data, writing and reviewing the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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