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Development of Novel Modeling Approaches for Rapid Risk Characterization of PFAS and PFAS Alternatives

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Per- and polyfluoroalkyl substances (PFAS) are persistent and pervasive chemicals frequently encountered by humans and wildlife. As a chemical class, PFAS include some 4000 different compounds making it difficult to adequately assess human risks due to PFAS exposure. As such, there are several data gaps, which often cannot be filled through traditional means for assessing exposure outcomes such as two-dimensional (2D) tissue culture on plastic or in vivo models. Organ-on-a-chip models and other full tissue models have become widely adopted as more accurate means for predicting human outcomes in response to exposure to drugs or compounds of interest. In this study, Emulate (Emulate, Inc.; Boston, MA) liver and renal proximal tubule chips were exposed to various concentrations of perfluorooctane sulfonic acid (PFOS) or perfluorohexane sulfonate (PFHxS) over time. Cytotoxic effects, which were different from those observed in 2D tissue culture, were detected because of exposures to both compounds. Phenotypes that may be detrimental to organ health after exposure to PFOS or PFHxS were also identified, such as changes in uric acid, albumin production and reactive oxygen species and reactive nitrogen species production compared with untreated chips. PFOS and PFHxS were detected in cell lysates and medium effluent over time.

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PREFACE

The work described in this report was authorized under the Applied Research for the Advancement of Science and Technology Priorities program element number 0602251D8Z through the Office of the Under Secretary of Defense Research and Engineering (Washington, DC). The work was started in May 2020 and completed in December 2021.

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EXECUTIVE SUMMARY

The work described in this technical report showcases the utility of the Emulate liver and renal proximal tubule kidney chips (Emulate, Inc.; Boston, MA) as surrogate models for assessing the toxicological effects of per- and polyfluoroalkyl substances (PFAS). Organ-on-a-chip models and other full tissue models have been gaining traction as more accurate means for predicting human outcomes in response to exposure to drugs or compounds of interest. Emulate has been successful in developing organ-on-a-chip technology that can reproduce the physiological and phenotypic characteristics of several different organs, including the liver, lung, intestine, kidney, and brain. The chips are composed of a top and bottom channel separated by a porous membrane, which houses the different cell types and microvasculature associated with the various organ types amenable to Emulate's system. In this study, Emulate liver and renal proximal tubule kidney chips were exposed to various concentrations of perfluorooctane sulfonic acid (PFOS) or perfluorohexane sulfonate (PFHxS) over time. Cytotoxic effects, different from those observed in two-dimensional tissue culture, were detected because of exposures to both compounds. Phenotypes that may be detrimental to organ health after exposure to PFOS or PFHxS were also identified and compared with untreated chips, such as changes in uric acid, albumin production, and reactive oxygen species/reactive nitrogen species production. PFOS and PFHxS were detected in cell lysates and medium effluent over time, providing an outlook on the pharmacodynamics properties of the compounds. These data highlight organ-on-a-chip technologies as viable means for generating predictions on the effects of PFAS compounds on human health.

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DEVELOPMENT OF NOVEL MODELING APPROACHES FOR RAPID RISK CHARACTERIZATION OF PFAS AND PFAS ALTERNATIVES

1. INTRODUCTION

1.1 Emulate Liver and Renal Proximal Tubule Kidney Organ Chips

Traditionally, researchers have relied heavily on two-dimensional tissue cultures and animal models to make toxicological predictions [1,2]; however, in vivo work is time consuming and has a high financial burden. More importantly, human hepatic tissue cultures grown in dishes and plates often lack physiological accuracy [3]. For these reasons, microphysiological systems (MPSs) have been gaining traction as more accurate and potentially more cost-effective means for predicting human outcomes in response to drug exposures or other compounds of interest [4,5]. By providing a three-dimensional model composed of a multi-cell type microenvironment complete with fluid flow and other cellular and environmental dynamics present in a functional organ, MPSs are leading the charge for alternative in vivo toxicity testing [6].

Recently, organ-on-a-chip technologies have successfully been shown to reproduce the physiological and phenotypic characterisitcs of many different organs, such as the brain, liver, lung, intestine, and kidney [4,6–11]. Each of these unique systems relies on specialized components and features that allow for organ mimicry, which surpass traditional tissue culture (Figure 1). With respect to the Emulate system (Emulate, Inc.; Boston, MA) used in this study, chips are composed of a top and bottom channel separated by a porous membrane, which houses the different cell types and microvasculature associated with the amenable organ types [5]. The liver chip and renal proximal tubule chip are designed to maintain hepatic- and kidney-specific cytoarchitecture as well as function. The chips are also subjected to constant, unilateral flow, which can be useful to both to facilitate exposures from exogenous compounds and provide the cells with the sheer stresses comparable to what would be experienced in vivo.

Four different cell types are maintained in the liver chips consisting of a layer of hepatocytes in the top channel, a layer composed of non-parenchymal cell (NPC) Kupffer cells and human stellate cells in the middle channel, and finally, a layer of liver sinusoidal endothelial cells (LSECs) in the bottom channel [5]. The renal proximal tubule chips are composed of renal proximal tubule epithelial cells in the top channel and a kidney endothelium in the bottom channel. The Emulate system consists of the Pods, Zoë, and Orb. The chips are housed in the Pods, which function to supply medium to the various cell types. Cells are dosed through the inlet resevoirs of the Pod, and effluent is collected from the outlet resevoirs for analysis. The flow rate of the medium through the top and bottom channels of the chip is controlled by the Zoë. The Zoë accomodates a pump manifold, which is engaged with the chips housed in the Pods to produce the medium flow. All of the gas exchange within the chips is controlled by the Orb [4,10,11].

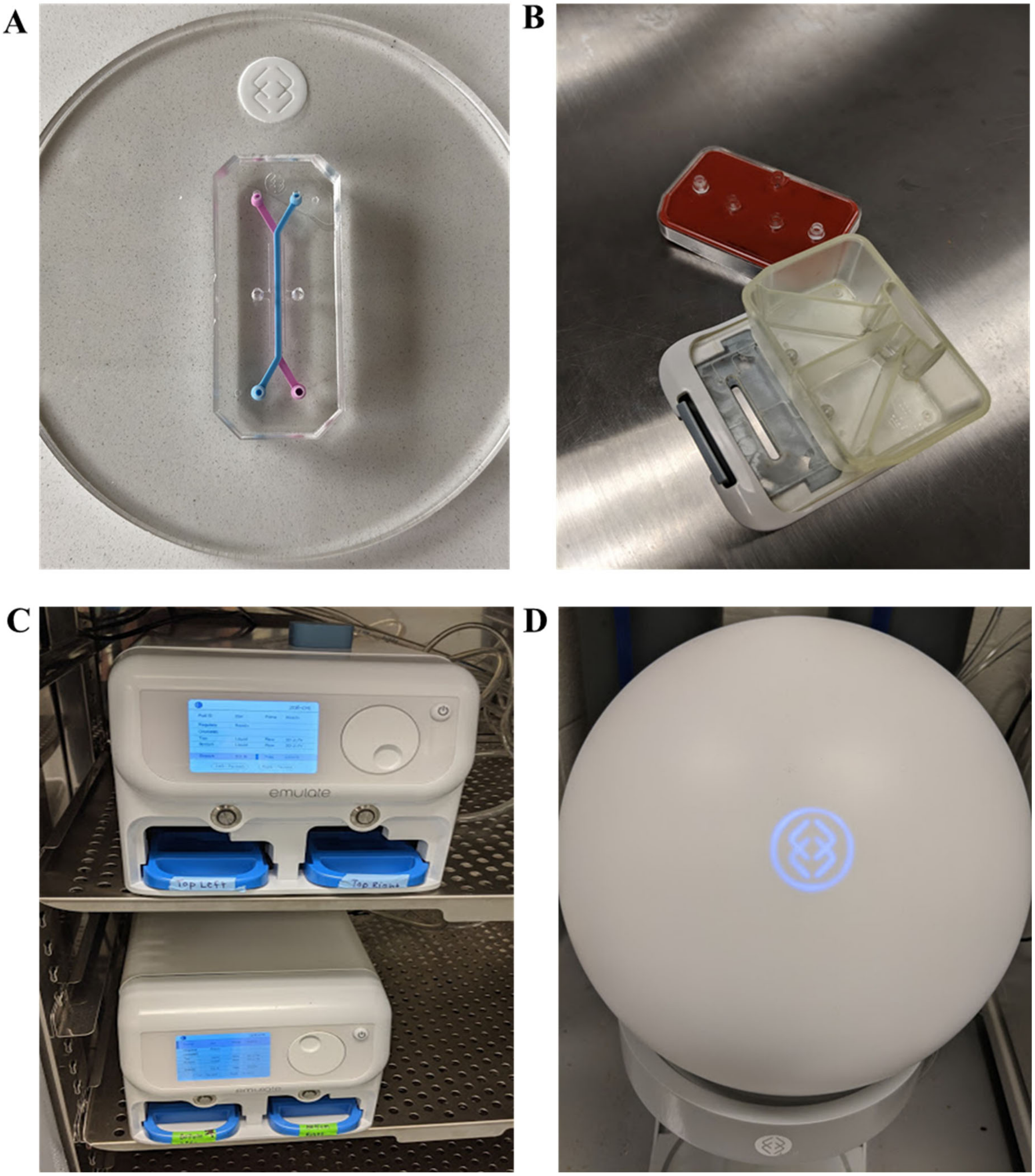

Figure 1. Components of the Emulate MPS: (A) the S1 chip, which consists of a top channel (blue) and a bottom channel (pink) where the various cells are seeded; (B) the Pod in which the chips are housed and which contains inlet and outlet reservoirs for medium; (C) the Zoë in which the Pods are placed and which controls the medium flow; (D) the Orb, which controls gas exchange.

1.2 Per- and Polyfluoroalkyl Substances (PFAS)

The PFAS group of synthetic chemical compounds contains multiple fluorine atoms attached to an alkyl chain (Figure 2). The earliest known form of these substances, polytetrafluoroethylene, was synthesized in 1938. It was registered under the trademark name, Teflon, by DuPont (Wilmington, DE) in 1945 [12]. Since then, PFAS have been used prolifically in industrial and consumer products and processes. [13]. Generally, PFAS have a prolonged half-life, which results in environmental persistence and contamination of food, water, and air [14]. Consequently, measurable blood levels of PFAS have been detected in up to >90% of the population of developed countries [14], and epidemiological studies have drawn correlations between exposure to PFAS and human health effects that include adverse reproductive and development outcomes like liver disease, kidney disease, altered immune function, and cancer [15].

When considering the widespread use of PFAS, their environmental persistence, and the current model systems used to assess PFAS toxicity, it becomes difficult to adequately assess mode of action and adverse outcome pathways [12]. This is primarily because humans have complex PFAS exposure profiles based on sources of exposure and geographical locations. Environmental conditions, such as seasonal patterns, rainfall, and proximity to industrial sites or military bases play important roles in population exposures. Secondly, it can be assumed that everyone born after 1950 has suffered some level of PFAS exposure. These reasons make it difficult to establish control populations for human studies. Finally, marked interspecies differences have been observed between experimental models for toxicological PFAS assessments. A human surrogate model capable of translating the gap between animals and humans is needed to better understand the health effects of PFAS exposure.

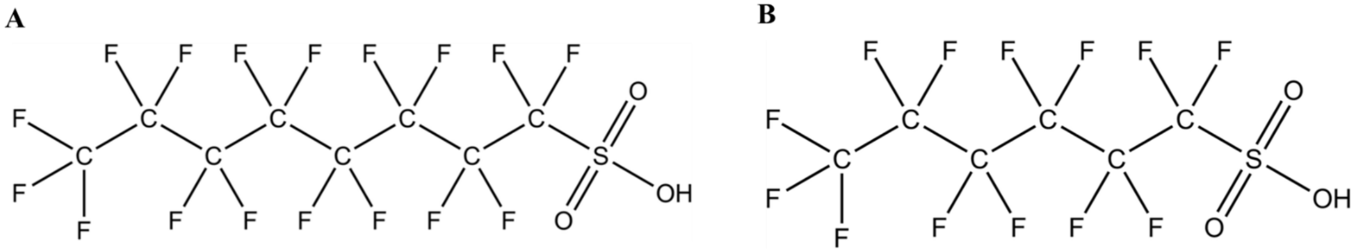

Figure 2. Chemical structure of (A) perfluorooctane sulfonic acid (PFOS) and (B) perfluorohexane sulfonate (PFHxS).

1.3 Projected Study Goals

To assess the data gaps surrounding PFAS toxicity and their effects on human health, we proposed using in vitro surrogates to animal models. In this study, Emulate liver and renal proximal tubule organ chips were exposed to various concentrations of perfluorooctane sulfonic acid (PFOS) and perfluorohexane sulfonate (PFHxS). Liver and kidney chips were chosen based on historical data suggesting toxicity and potential compound retention in these two organs. Our aim was to generate human relevant data that translate to the toxicological effects seen in humans, thus further validating human organ chip systems as viable alternatives to traditional in vivo toxicity studies. Because studies using organ chip systems are generally more cost effective and enable higher throughput screening than animal studies, the platforms described in this report could be leveraged to study the toxicological effects of additional PFAS compounds and exposure paradigms.

2. METHODOLOGY

2.1 Materials and Chemicals

The following list shows the materials and chemicals that were used in this study together with their manufacturers:

- liver biokit (Emulate): the kit includes 24 S1 chips, human hepatocytes, human HSCs, human Kupffer cells, LSECs, and epitope retrieval (ER)-1 and ER-2 solutions;
- kidney renal proximal tubule biokit (Emulate): the kit includes 24 S1 chips, human renal proximal tubule epithelial cells (hRPTECs), human glomerular microvascular endothelial cells (hGMVECs), and ER-1 and ER-2 solutions;
- Dulbecco’s phosphate-buffered saline without Ca^{2+} and Mg^{2+} (DPBS; Corning; Corning, NY);
- 10× DPBS without Ca^{2+} and Mg^{2+} (Corning);
- trypan blue (MilliporeSigma; Burlington, MA);
- Percoll solution (MilliporeSigma);
- trypsin-ethylenediaminetetraacetic acid (EDTA; MilliporeSigma);
- William’s E Medium (WEM) and phenol red (MilliporeSigma);
- WEM, no phenol red (MilliporeSigma);
- Cell Systems Corporation (CSC; Kirkland, WA**)** medium kit: the kit includes CSC medium, culture boost, attachment factor, and cell freezing medium;
- renal epithelial basal medium (REBM; Lonza; Morristown, NJ);
- renal epithelial cell growth medium SingleQuots kit (REGM; Lonza)
- Matrigel extracellular matrix (ECM; Corning);
- fibronectin (Thermo Fisher Scientific; Waltham, MA);
- collagen type I (Corning);
- collagen IV (Corning);
- penicillin–streptomycin (MilliporeSigma);
- GlutaMax supplement (Thermo Fisher Scientific);
- L-ascorbic acid (MilliporeSigma);
- dexamethasone (MilliporeSigma);
- fetal bovine serum (FBS; MilliporeSigma);
- ITS+ premix universal culture supplement (Corning);
- human uric acid assay kit (Abcam; Waltham, MA);

- human albumin enzyme-linked immunosorbent assay (ELISA) kit (Abcam);
- CyQUANT lactate dehydrogenase (LDH) cytotoxicity assay (Thermo Fisher Scientific); and
- OxiSelect in vitro and reactive oxygen species/reactive nitrogen species (ROS/RNS) assay kit (Cell Biolabs).

2.2 Cell Storage

Human hepatocytes (HSCs), Kupffer cells, LSECs, hRPTECs, and hGMVECs were purchased directly from Emulate. The cells were kept frozen in liquid nitrogen storage (vapor phase) until they were cultured.

2.3 Reagent Preparation

All reagents were aliquoted at appropriate volumes prior to use to avoid multiple freeze–thaw cycles. Fibronectin was resuspended in cell culture grade water to a concentration of 1 mg/mL. Matrigel was thawed overnight on slushy ice in a 4 °C refrigerator. Using cold pipette tips, Matrigel was aliquoted to 5 mg aliquots based on the specific stock concentration. L-ascorbic acid was resuspended in cell culture grade water to a concentration of 50 mg/mL. Dexamethasone was resuspended in cell culture grade dimethyl sulfoxide (DMSO) at concentrations of 1 mM and 10 mM. All reagent aliquots were stored at –20 °C. PFOS and PFHxS stock solutions were prepared by dissolving compound in high-performance liquid chromatography (HPLC) grade H_2O at a concentration of 1 mg/mL with stirring overnight protected from light.

The following media preparations were used in this study:

- base LSEC culture medium (500 mL):
  485 mL of CSC basal medium, 10 mL of culture-boost, and 5 mL of penicillin–streptomycin;

- complete LSEC culture medium (50 mL):
  45 mL of base LSEC culture medium and 5 mL of FBS;

- base hepatocyte seeding medium (500 mL):
  490 mL WEM (with phenol red), 5 mL of penicillin–streptomycin, and 5 mL of GlutaMax;

- complete hepatocyte seeding medium (200 mL):
  187.78 mL of base hepatocyte seeding medium, 2 mL of ITS+ premix, 200 µL ascorbic acid, 20 µL of dexamethasone (10 mM), and 10 mL of FBS;

- base hepatocyte maintenance medium (500 mL):
  490 mL of WEM (without phenol red), 5 mL of penicillin–streptomycin, and 5 mL ofGlutaMax;

- complete haptocyte maintenance medium (50 mL):
  49.445 mL of base hepatocyte maintenance medium, 500 µL of ITS+ premix, 50 µL of ascorbic acid, and 5 µL of dexamethasone (1mM);

- hepatocyte overlay medium:
  19.5 mL of complete hepatocyte maintenance medium and 0.5 mL of Matrigel;

- NPC seeding medium (50 mL):
  22.5 mL of complete hepatocyte maintenance medium omitting dexamethasone, 22.5 mL of base LSEC culture medium, and 5 mL of FBS;

- NPC maintenance medium (50 mL):
  24.5 mL of complete hepatocyte maintenance medium omitting dexamethasone, 24.5 mL base LSEC culture medium, and 1 mL of FBS;

- NPC maintenance medium (50 mL):
  24.5 mL of complete hepatocyte maintenance medium omitting dexamethasone, 24.5 mL of base LSEC culture medium, and 1 mL of FBS;

- base human glomerular microvascular endothelial cell (hGMVEC) culture medium (500 mL):
  485 mL of CSC basal medium, 10 mL culture-boost, and 5 mL of penicillin–streptomycin;

- complete hGMVEC culture medium (50 mL):
  45 mL of base hGMVEC culture medium, and 5 mL of FBS;

- complete hGMVEC maintenance medium (50 mL):
  49.75 mL of base hGMVEC culture medium and 0.25 mL of FBS;

- base human renal proximal tubule epithelial cell (hRPTEC) culture medium (500 mL):
  492 mL of REBM and REGM (3 mL total) SingleQuots kit and 5 mL of penicillin–streptomycin;

- complete hRPTEC culture maintenance medium (50 mL):
  49.75 mL of base hRPTEC culture medium and 0.25 mL of FBS.

2.4 LSEC Culture

LSECs were removed from liquid nitrogen storage and quick-thawed by placing the cryovial in a 37 °C water bath. The contents of the vial were transferred to a 15 mL conical tube containing 3 mL of warm complete LSEC culture medium. The vial was rinsed once with 1 mL of medium, and the 15 mL conical tube was brought up to a volume of 15 mL of complete LSEC culture medium. The cell solution was centrifuged at 200 ×g for 5 min at room temperature. The supernatant was aspirated, and the cells were resuspended in 15 mL of fresh complete LSEC culture medium. The LSEC suspension was added to a T-75 flask that was pre-coated with attachment factor and incubated at 37 °C and 5% CO_2. LSECs were cultured at least two days before chip seeding, and medium was exchanged every two days.

2.5 hGMVEC Culture

hGMVECs were removed from liquid nitrogen storage and quick-thawed by placing the cryovial in a 37 °C water bath. The contents of the vial were transferred to a 15 mL conical tube containing 3 mL of warm complete hGMVEC culture medium. The vial was rinsed once with 1 mL of medium, and the 15 mL conical tube was brought up to a volume of 15 mL of complete hGMVEC culture medium. The cell solution was centrifuged at 200 xg for 5 min at room temperature. The supernatant was aspirated, and the cells were resuspended in 15 mL of fresh complete hGMVEC culture medium. The hGMVEC suspension was added to a T-75 flask that was pre-coated with attachment factor and incubated at 37 °C and 5 % CO_2. hGMVECs were cultured at least two days before chip seeding, and medium was exchanged every two-days.

2.6 hRPTEC Culture

hRPTECs were removed from liquid nitrogen storage and quick-thawed by placing the cryovial in a 37 °C water bath. The contents of the vial were transferred to a 15 mL conical tube containing 3 mL of warm complete hGMVEC culture medium. The vial was rinsed once with 1 mL of medium, and the 15 mL conical tube was brought up to a volume of 15 mL of complete hGMVEC culture medium. The hGMVEC suspension was added to a T-75 flask and incubated at 37 °C and 5 % CO_2. hGMVECs were cultured at least two days before chip seeding, and medium was exchanged every two days.

2.7 Chip Activation

ER-1 and ER-2 reagents are light sensitive, so all manipulations were performed in a bio-safety cabinet with the lights turned off. Before preparation of the activation solution, ER-1 and ER-2 reagents were allowed to equilibrate to room temperature. Then, 1 mL of ER-2 was added to the ER-1 vial, and the contents were added directly to a 15 mL conical tube. An additional 1 mL of ER-2 was added to the ER-1 vial to collect remaining material, and that was added to the 15 mL conical tube. This was repeated two more times. Next, 6 mL of ER-2 was added to 4 mL of ER-1 solution in the 15 mL conical tube for a final concentration of

0.5 mg/mL. Using a p200 pipette, the ER-1 solution was introduced to the top and bottom channels of the S1-chip at a volume of 50 µL and 20 µL, respectively, with care so as not to introduce bubbles. The excess solution was aspirated from the top of the chip. The chips were activated under constant UV light for 20 min. After UV activation, the ER-1 solution was aspirated from both channels, and both channels were washed with 200 µL ER-2 solution. The top and bottom channels of each chip were washed with 200 µL of 1× DPBS, and cold 1× DBPS was left in the channel. The chips were incubated at 4 °C overnight.

2.8 ECM Coating

The liver chip ECM was prepared on ice by combining collagen type I and Matrigel in ice cold 1× DPBS at a concentration of 100 µg/mL and 25 µg/mL, respectively. After ECM preparation, the cold 1× DPBS was removed from the top and bottom channels of the chips. ECM was added to each channel using a p200 pipette until small droplets formed on the channel outlets. Droplets of ECM were placed on all four ports of each chip, and the chips were incubated at 4 °C overnight.

The renal proximal tubule chip ECM was prepared on ice by combining collagen IV and fibronectin in ice cold 1× DPBS at concentrations of 50 and 100 µg/mL, respectively. After ECM preparation, the cold 1× DPBS was removed from the top and bottom channels of the chips. ECM was added to each channel using a p200 pipette until small droplets formed on the channel outlets. Droplets of ECM was placed on all four ports of each chip, and the chips were incubated at 4 °C overnight.

2.9 Liver Chip Hepatocyte Seeding

The liver chips were equilibrated to room temperature and washed three times with 200 µL of complete hepatocyte seeding media. The final wash was left in the top and bottom channels of the chips. Human hepatocytes were removed from liquid nitrogen storage, and the cryovial was quick-thawed in a 37 °C water bath. The thawed cell solution was quickly added to 3 mL of warm complete hepatocyte seeding medium in a 50 mL conical tube. The vial was rinsed with 1 mL of medium, which was then transferred to the 50 mL conical tube. Complete hepatocyte seeding medium was slowly added to the 50 mL conical tube until the volume was brought to 35 mL. Then, 15 mL of 90% Percoll solution in 1× DPBS was slowly layered on top of the 35 mL of cell solution. The cells were centrifuged at 96 ×g for 6 min at room temperature. The supernatant was carefully aspirated until 3–5 mL remained, and the pellet was undisturbed. The pellet was gently resuspended by tilting and rotating the 50 mL conical tube. Complete hepatocyte seeding medium was added to the 50 mL conical tube until the total volume was 50 mL, and the cell solution was centrifuged at 72 ×g for 4 min at room temperature. The supernatant was carefully removed until the volume was down to 1–2 mL, and the pellet was carefully resuspended as described above. The human hepatocytes were counted in trypan blue solution using a hemocytometer and resuspended to a final cell density of 3.5×10^6 cells/mL in complete hepatocyte seeding medium. Next, 50 µL of the cell suspension was carefully added to the top channel of each chip. The chips were incubated at 37 °C with 5% CO_2 for 3 h, or until the hepatocytes attached. After they attached, a gravity wash was performed on each chip by gently dropping 200 µL of complete hepatocyte seeding medium on top of both inlet ports of the top and bottom channels. The chips were incubated overnight at 37 °C with 5% CO_2.

2.10 Hepatocyte Matrigel Overlay

Matrigel was slowly thawed on slushy ice. Preparation of the hepatocyte overlay medium was also done on ice, which was prepared by diluting the Matrigel into ice cold complete hepatocyte maintenance medium with cold tips to a concentration of 250 µg/mL. The chips were removed from the incubator and washed with 200 µL of complete hepatocyte maintenance medium. After that, 200 µL of complete hepatocyte overlay medium was pipetted into the top channel of each tip, leaving droplets on both the inlets and the outlets. The chips were incubated overnight at 37 °C and 5% CO_2.

2.11 Liver Chip NPC Seeding

The LSECs were harvested from the T-75 flask using 3 mL of trypsin-EDTA. Next, 9 mL of warm NPC seeding medium was added to the cell suspension, and the entire 12 mL was centrifuged in a 15 mL conical at 200 ×g for 5 min. The supernatant was aspirated, and100 µL was left in the tube for resuspending the LSECs. The LSECs were counted in trypan blue solution using a hemocytometer and resuspended to a cell density of 9×10^6 cells/mL in cold NPC seeding medium. The cell suspension was kept on ice while the other cell types were prepared for seeding. HSCs were removed from liquid nitrogen storage, and the cryovial was quick-thawed in a 37 °C water bath. The thawed cell solution was quickly added to 3 mL of warm NPC seeding medium in a 15 mL conical tube. The vial was rinsed with 1 mL of medium, and transferred to the 15 mL conical tube. The cell suspension was increased to 15 mL with NPC seeding medium and centrifuged at 250 ×g for 5 min. The supernatant was aspirated, and 100 µL was left in the tube for resuspending the HSCs. The cells were counted in trypan blue solution using a hemocytometer, resuspended to a cell density of 0.3×10^6 cells/mL in cold NPC seeding medium, and placed on ice. Kupffer cells were removed from liquid nitrogen storage and the cryovial was quick-thawed in a 37 °C water bath. The thawed cell solution was quickly added to 3 mL of warm NPC seeding medium in a 15 mL conical tube. The vial was rinsed with 1 mL of medium, and transferred to the 15 mL conical tube. The cell suspension was increased to 15 mL with NPC seeding medium and centrifuged at 250 × g for 5 min. The supernatant was aspirated, and 100 µL was left in the tube for resuspending the Kupffer cells. The cells were counted in trypan blue solution using a hemocytometer, resuspended to a cell density of 1.5×10^6 cells/mL in cold NPC seeding medium. The 3 NPC cell suspensions were mixed in a 1:1:1 ratio (v/v/v) in a 15 mL conical tube on ice, and 20 µL of the combined NPC suspension were added to the bottom channel of each chip. After seeding, the chips were inverted and incubated at 37 °C and 5% CO_2 for 4 h or until the cells attached. After the cells were attached to the bottom channel, a gravity wash was performed with 200 µL of hepatocyte maintenance medium for the top channel and NPC seeding medium for the bottom channel. The chips were incubated overnight at 37 °C and 5% CO_2.

2.12 Renal Proximal Tubule Chip hRPTEC Seeding

The hRPTECs were harvested from the T-75 using 3 mL of trypsin-EDTA. Then, 9 mL of warm hRPTEC culture medium was added to the cell suspension, and the entire 12 mL was centrifuged in a 15 mL conical at 200 ×g for 5 min. The supernatant was aspirated, and 100 µL was left in the tube to resuspend the hRPTECs. The hRPTECs were counted in trypan blue solution using a hemocytometer and resuspended to a cell density of 1×10^6 cells/mL in warm hRPTEC culture medium. Then, 35–50 µL of the hRPTEC suspension was added to the top channel of each chip. After the cells were attached to the top channel, a gravity wash was performed with 200 µL of hRPTEC culture medium for the top channel. The chips were incubated overnight at 37 °C and 5% CO_2.

2.13 Renal Proximal Tubule Chip hGMVEC Seeding

The hGMVECs were harvested from the T-75 flask using 3 mL of trypsin-EDTA, and 9 mL of warm hGMVEC culture medium was added to the cell suspension. Next, the entire amount (12 mL) was centrifuged in a 15 mL conical tube at 200 ×g for 5 min. The supernatant was aspirated, leaving 100 uL in the tube with which to resuspend the hGMVECs. The hGMVECs were counted in trypan blue solution using a hemocytometer and resuspended to a cell density of 3×10^6 cells/mL in warm hGMVEC culture medium. Around 15–25 µL of the hGMVEC suspension was added to the bottom channel of each chip. After seeding, the chips were inverted and incubated at 37 °C and 5% CO_2 for 4 h or until the cells attached. After the cells were attached to the bottom channel, a gravity wash was performed with 200 µL of hGMVEC culture medium. The chips were incubated overnight at 37 °C and 5% CO_2.

2.14 Chips to Pods and Pods to Zoë

Complete hepatocyte maintenance medium, NPC maintenance medium, hRPTEC maintenance medium, and hGMVEC maintenance medium were warmed in a 37 °C water bath for 1 h. To reduce the risk for bubbles in the chips, the medium used for the Pods was gas equilibrated in 50 mL steriflip conical tubes for 5 min before use. After that, 300 µL of complete hepatocyte maintenance medium or hRPTEC maintenance medium was added directly over the via (microfluidic channel opening) of the top channel outlet reservoir via, and 300 µL of NPC maintenance medium or hGMVEC maintenance medium was added to the bottom channel outlet reservoir in the same manner. Then, 3 mL of the appropriate medium was added to the inlet reservoirs of each Pod. The Pods were inserted into the Zoë and primed. After priming, the top and bottom channels of the chips were washed with 200 µL of the appropriate medium and were manually connected to each Pod. The Pods with chips were then replaced into the Zoë, and a regulate cycle was run. After the cycle was completed, the flow conditions were changed to 30 µL/h for both top and bottom channels. The top and bottom inlet reservoir medium was replenished every two days while the chips were under flow.

2.15 PFOS/PFHxS Solution Preparation and Dosing

PFOS and PFHxS working solutions were prepared by diluting the compounds in NPC maintenance medium or hGMVEC maintenance medium to the appropriate final concentration used in a particular assay for this study. A vehicle control was also prepared by diluting cell culture grade DMSO in NPC maintenance medium or hGMVEC maintenance medium to a final concentration of 0.1%. To expose the chips, bottom channel inlet reservoir medium was aspirated and replaced with 3 mL of medium containing either PFOS, PFHxS, or the DMSO vehicle control so that the compounds would be introduced on the endothelial channel. The Pods were returned to the Zoë, and the PFOS or PFHxS exposures were initiated by increasing the flow rate of the Zoë to 600 µL/h for 5 min. The flow rate was then returned to 30 µL/h. After exposure, effluent was collected from the top channel outlet reservoir for analysis. Cell lysates were also prepared for downstream analysis by washing the top channels of the chips with 50 µL of radioimmunoprecipitation assay buffer for 2 min. The cell lysate was collected for analysis.

2.16 Human Albumin ELISA

The albumin ELISA was performed in accordance with the protocol set out in Abcam's human albumin ELISA assay kit. All endpoint optical densities (ODs) were read on a FlexStation III multimode plate reader (Richmond Scientific; Lancashire, UK) at a wavelength of 450 nm.

2.17 Human Uric Acid Assay

The colorimetric uric acid assay was performed in accordance with the protocol set out in Abcam's human uric acid assay kit. All endpoint ODs were read on a FlexStation III (Molecular Devices; San Jose, CA) multimode plate reader at a wavelength of 570 nm.

2.18 LDH Cytotoxicity Assay

The LDH cytotoxicity assay was performed in accordance with the protocol set out in CyQUANT LDH cytotoxicity assay. All endpoint ODs were read on a FlexStation III multimode plate reader, at wavelengths of 490 and 680 nm.

2.19 ROS and RNS Assay

The ROS/RNS assay was performed in accordance with the protocol set out in the OxiSelect in vitro ROS/RNS assay kit. All endpoint ODs were read on a FlexStation III multimode plate reader at wavelengths of 480 and 530 nm.

2.20 Mass Spectrometry (MS) PFOS and PFHxS Analysis

A calibration curve was created using 50 µg/mL stocks of PFOS and PFHxS (Cambridge Isotope Laboratories, Inc.; Tewksbury, MA) in H_2O and 50 µg/mL stocks of stable isotope labeled $^{13}C_6$-PFOS and $^{13}C_8$-PFHxS in methanol. A 1:100 dilution was made to result in a 0.5 µg/mL solution (2 µL stock standard, 198 µL H_2O). A four standard mix solution was made from the 0.5 µg/mL stocks. This contained 40 µL of 2.0 ug/mL PFOS, PFHxS, PFOS labeled, and PFHxS labeled. A calibration curve with 10 concentrations was made using PFOS, PFOS unlabeled, PFHxS, and PFHxS unlabeled. The different concentrations were 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, and 0.0001 µg/mL. A 20 µL of liver and renal proximal tubule effluents or lysates were aliquoted and mixed with 20 µL of internal standard (1 µg/mL 50:50 PFOS:PFHxS in H_2O). Both calibration curve and liver and renal proximal tubule effluents or lysates were analyzed on an Q Exactive Plus mass spectrometer (Thermo Fisher Scientific). A 2 µL of sample was injected into a 2.1 × 150 mm, 2.7 µm CORTECS T3 column on a Vanquish HPLC (Thermo Fisher Scientific) with buffer A of water with 0.1% formic acid and buffer B of acetonitrile with 0.1% formic acid. The samples were run for 7 min at 0.350 mL/min with the following reverse phase gradient: 0–6 min 50% B, 6–6.5 min 90% B, and 6.5–7 min 10% B. The MS method included a 2.5–7 min parallel reaction monitoring (PRM) run. Samples were analyzed in PRM from 2.48 to 7 min in negative ion mode in a charge state of 2 with inclusion turned on. The samples were also analyzed using tandem MS at a resolution of 17,5000, AGC target of 2 × 10^5, maximum injection time of 100 m/s, isolation window of 4.0 m/z, and collision energy of 35.

3. RESULTS

3.1 Assessing PFOS/PFHxS Cytotoxicity

HepG2 liver cells and human embryonic kidney (HEK) cells were exposed to various concentrations of PFOS or PFHxS for 48 h to determine a suitable, sublethal concentration range for the liver and renal proximal tubule chip exposures (Figure 3). The assay determined higher concentrations of both PFHxS and PFOS to be lethal to HepG2 cells (Figure 3A), whereas PFOS was more lethal than PFHxS for HEK cells (Figure 3B). From these data, it was determined that the chips would be exposed to two-fold dilutions of PFOS and PFHxS starting at 50 µM (25 µg/mL and 20 µg/mL, respectively). PFOS was found to be partially cytotoxic in liver chips at 25 µg/mL, with PFHxS causing negligible cytotoxicity in liver (Figure 4A). Both compounds were observed to be more cytotoxic in the kidney chips at the highest concentrations tested but still within an acceptable range for sub lethality (Figure 4B).

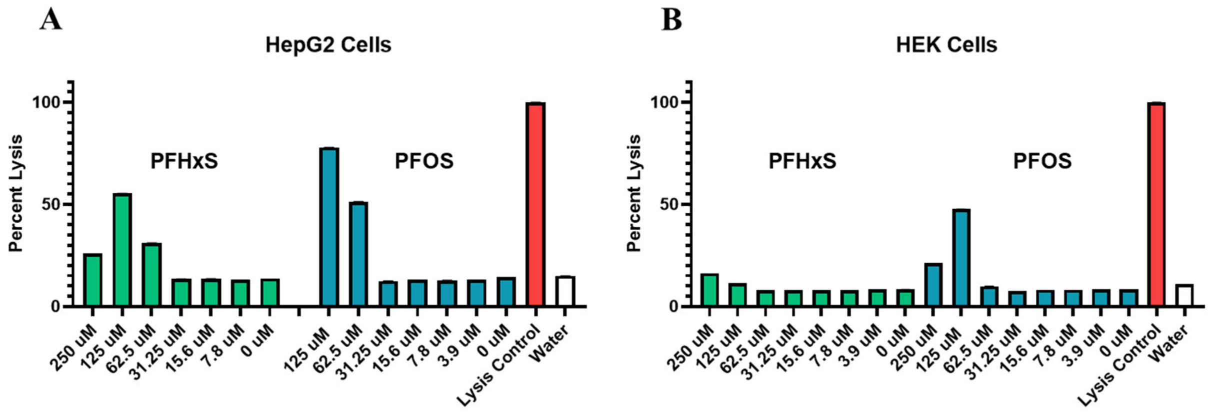

Figure 3. LDH cytotoxicity assay showing the effects of PFOS and PFHxS on (A) HepG2 liver cells (B) and HEK cells after 48 h. Each bar represents the average cytotoxicity of three individual wells with the error bars indicating standard deviations. Lysis was determined as a percentage of the lysis control wells.

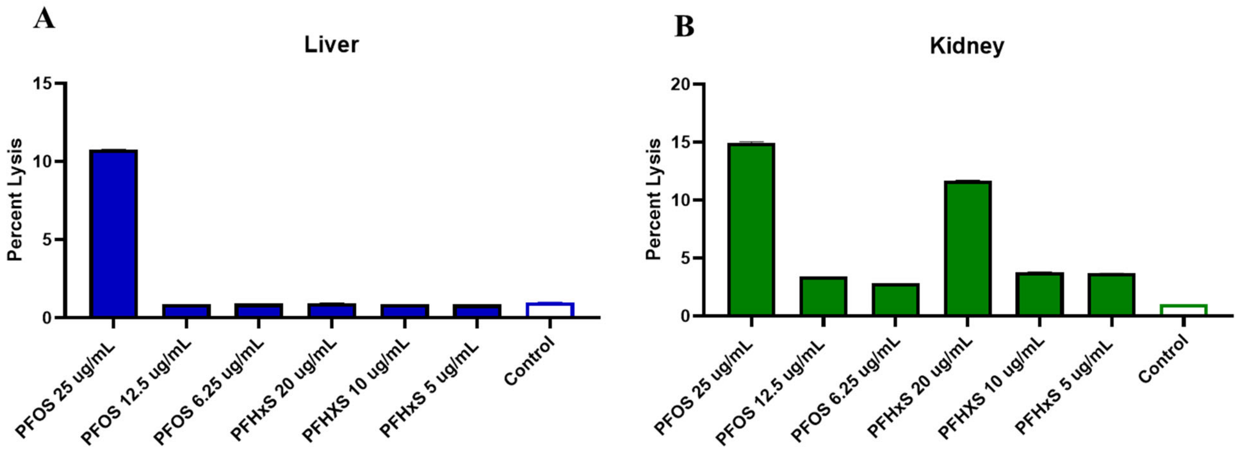

Figure 4. LDH cytotoxicity assay showing the effects of PFOS and PFHxS on (A) liver chips and (B) renal proximal tubule kidney chips after 48 h. Each bar represents the average cytotoxicity of three individual chips, with the error bars indicating standard deviations. Lysis was determined as a percentage of the lysis control wells.

3.2 Liver Albumin Production

Albumin ELISA assays were performed on liver chip effluent after 24 h of exposure. These indicated that albumin production was equal to or greater than that of the untreated chips (Figure 5). It is interesting to note that albumin is the largest carrier protein for both PFOS and PFHxS in native human plasma [16]. The data here suggest that exposure to PFOS or PFHxS at the concentrations tested has no deleterious effect on hepatic function in the context of albumin production.

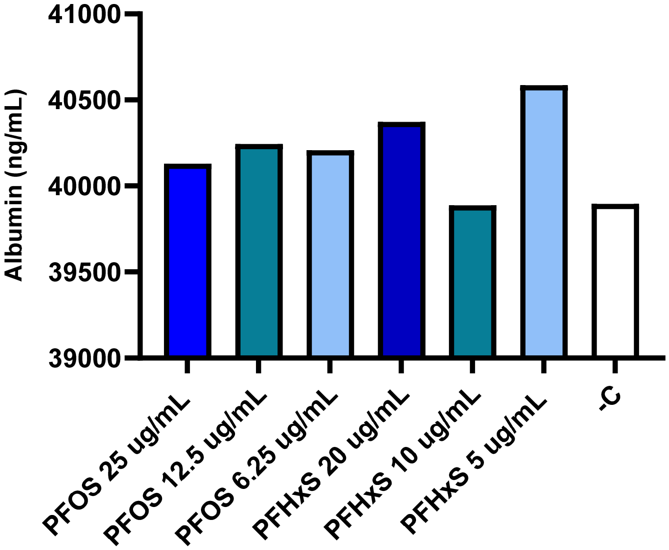

Figure 5. Human albumin ELISA assay performed on liver chip effluent collected from the top channel outlet reservoir 24 h after PFOS or PFHxS exposure. Each bar represents the average albumin production of three individual chips, with the error bars indicating standard deviations. The concentrations were determined by standard curve.

3.3 Uric Acid Production

Uric acid colorimetric assays were performed on liver chip and kidney chip effluent after 24 h of exposure (Figure 6). Uric acid concentrations were considerably lower in the liver chip effluent compared with those of the untreated vehicle control. This is associated with liver disease in humans. Hyperuricemia, which was observed in the highest concentration of PFHxS in the kidney chips, results in the formation of urate, which subsequently leads to gout or kidney disease.

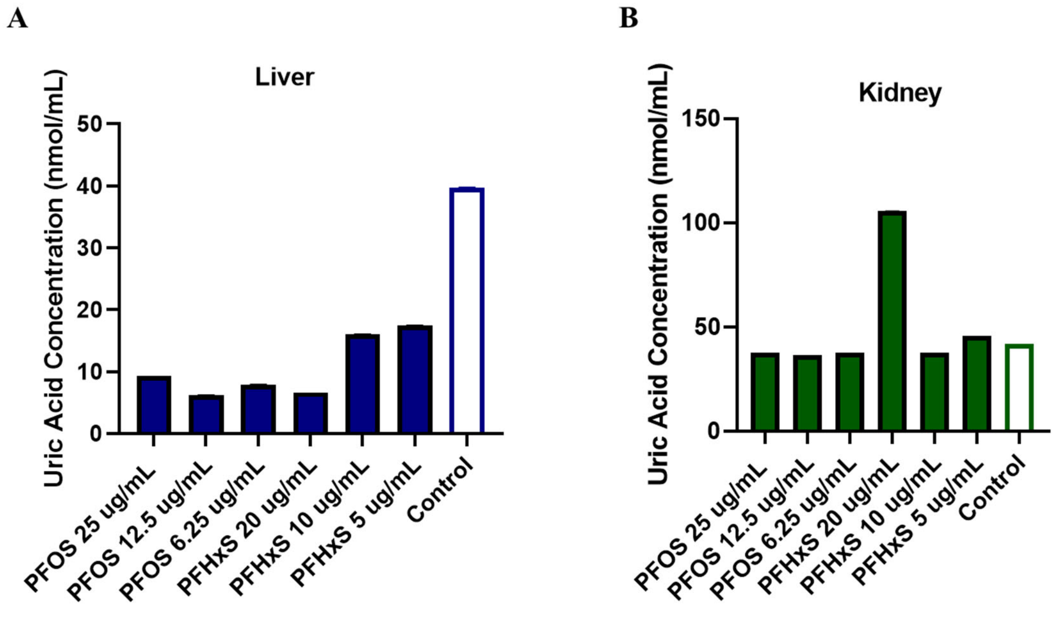

Figure 6. Human uric acid colorimetric assay performed on (A) liver chip effluent and (B) renal proximal tubule kidney chip effluent collected from the top channel outlet reservoir 24 h after PFOS or PFHxS exposure. Each bar represents the average uric acid production of three individual chips, with the error bars indicating standard deviations. The concentrations were determined by standard curve.

3.4 ROS/RNS Production

In vitro ROS/RNS assays were performed on liver chip and kidney chip effluent 3, 6, and 24 h post exposure (Figure 7). A dramatic decrease in ROS/RNS was observed 6 h post exposure to PFOS and PFHxS in liver chips compared with that of the untreated vehicle control (Figure 7A). These data contrast with previous in vitro studies, which show that these compounds increase ROS production in HepG2 cells [17].

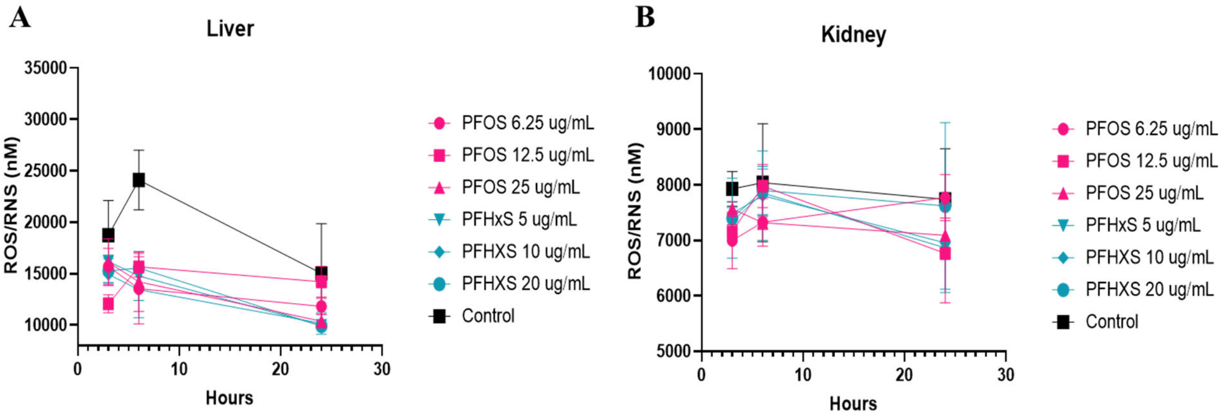

Figure 7. ROS/RNS detection assay performed on (A) liver chip effluent and (B) renal proximal tubule kidney chip effluent collected from the top channel outlet reservoir 3, 6, and 24 h after PFOS or PFHxS exposure. Each point represents the average ROS/RNS production of three individual chips, with the error bars indicating standard deviations.

3.5 PFOS/PFHxS Retention in Tissue and Effluent

Effluent medium collected from the top channel outlet reservoir of liver and kidney chips was analyzed by MS for the presence of PFOS or PFHxS over time (Figure 8). Both compounds were detected in liver effluent in a dose-dependent manner with increasing amounts recovered from 3 to 24 h post exposure. Full recovery of the initial dose concentrations was not observed by 24 h for either compound in the liver chips (Figure 8A, B), suggesting that the cell barrier was preventing the compounds from crossing into the top channel medium or that the compounds were taken up into the hepatocytes. More PFHxS was detected in the kidney effluent than the liver effluent, achieving 100% recovery at 10 µg/mL after 24 h of exposure (Figure 8D). However, there was a significant amount of PFOS detected after 6 h (Figure 8C), which deviated from the trend of increasing concentrations over time seen in the liver exposures and the PFHxS kidney exposure.

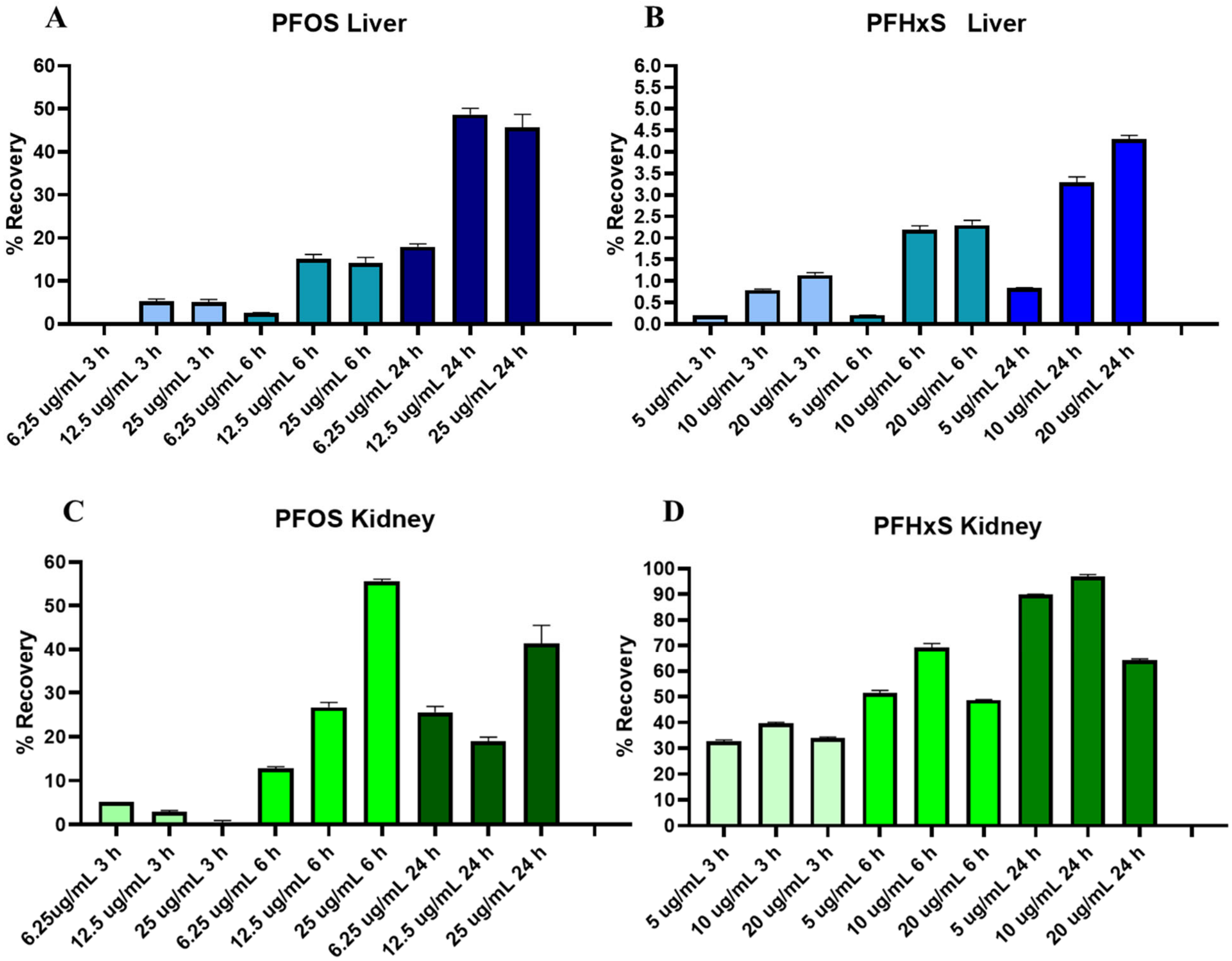

Figure 8. Effluent was collected at 3, 6, and 24 h post PFHxS or PFOS exposures from (A, B) liver chips and (C, D) renal proximal tubule kidney chips. Detection of the compounds was analyzed on a Q Exactive Plus mass spectrometer. Each bar represents the average compound percentage of three individual chips, with the error bars indicating standard deviations. Recovery was determined as a percentage of the initial exposure concentrations received by each chip.

After 48 h post-exposure of PFOS or PFHxS, human hepatocytes and RPTECs were lysed from the chips and analyzed for cellular compound retention (Figure 9). Less than 1% of PFHxS was detected in the liver cells at all concentrations tested, whereas 20.9% of the initially dosed 25 µg/mL concentration of PFOS was recovered. All (100%) of the PFHxS was recovered from kidney lysates at the 10 µg/mL dose; however, less than half was recovered at the highest dose. PFOS was recovered from kidney lysates in a dose-dependent manner, with 38.6% detected at the highest dose.

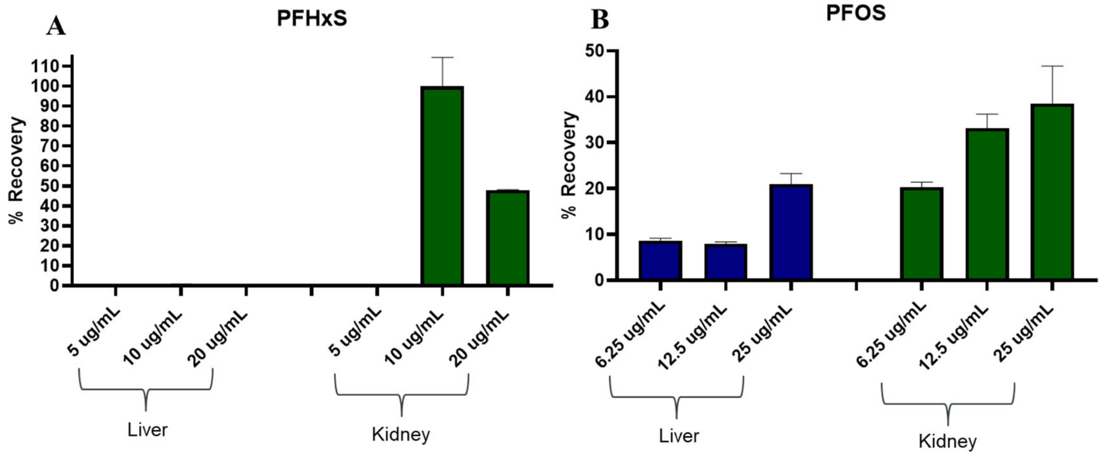

Figure 9. Liver and kidney cell lysates were collected 48 h post exposure to (A) PFHxS or (B) PFOS. Detection of the compounds was analyzed on an Q Exactive Plus mass spectrometer. Each bar represents the average compound percentage of 3 individual chips, with the error bars indicating standard deviations. Recovery was determined as a percentage of the initial exposure concentrations received by each chip.

4. CONCLUSIONS

The work described in this report provides insight into the utility of micro-physiological systems as an effective in vitro model for assessing the toxicological effects of PFAS substances on human tissues, such as those derived from the liver and kidney. Because the chips are under constant microfluidic flow, the longevity of primary cell types is increased dramatically. This allows for the assessment of long-term toxicity of agents and compounds of interest. Furthermore, the flow also allows for continual tissue exposure to the compound over time.

In this study, we were able to demonstrate cytotoxic effects of constant PFOS and PFHxS 48 h after the cells had already been established in the chips for over a week. Chip exposures elucidated phenotypes that may be detrimental to organ health after exposure to PFOS or PFHxS, such as changes in uric acid production. PFOS and PFHxS effluent and lysate retention data provide valuable insight into the pharmacokinetic properties of the compounds in both organ systems, as well as an understanding on organ tissue uptake. These data corroborate what is already known concerning PFAS retention in human organs and organ chip model systems, such as the Emulate liver and renal proximal tubule kidney chips. These data also allow researchers to further elucidate chronic toxicity without relying heavily on in vivo studies.

It should also be noted that both PFOS and PFHxS exposure for 6 h resulted in a sharp decrease in ROS/RNS production in liver chips, which is contrary to published data derived from immortalized cell lines where ROS/RNS production increased. This difference could be explained by the physiological differences between the healthy liver chip tissues and the HepG2 liver cancer cell line, but this discrepancy should be investigated further.

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ACRONYMS AND ABBREVIATIONS

CSC	Cell Systems Corporation
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	epitope retrieval
FBS	fetal bovine serum
HEK	human embryonic kidney
hGMVEC	human glomerular microvascular endothelial cell
HPLC	high-performance liquid chromatography
hRPTEC	human renal proximal tubule epithelial cell
LDH	lactate dehydrogenase
LSEC	liver sinusoidal endothelial cells
MPS	microphysiological system
MS	mass spectrometry
NPC	non-parenchymal cell
OD	optical density
PRM	parallel reaction monitoring
PFAS	per- and polyfluoroalkyl substances
PFHxS	perfluorohexane sulfonate
PFOS	perfluorooctane sulfonic acid
REBM	renal epithelial basal medium
REGM	renal epithelial cell growth medium
RNS	reactive nitrogen species
ROS	reactive oxygen species
WEM	William's E Medium

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